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AN UNSERE EINSENDER

(Für allgemeine Auskunft siehe die Rückseite des Umschlags)

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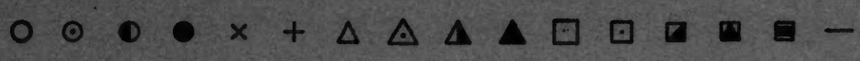

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I. M. BETT UND G. P. FRASER, *Clin. Chim. Acta*, 4 (1959) 346.

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T. MOORE, *Vitamin A*, Elsevier, Amsterdam, 1957, p. 243.

Zusammenfassung

Der Inhalt des Artikels soll in 50 bis 200 Worten zusammengefasst werden. Es ist erwünscht, dass deutsche und französische Beiträge auch mit der englischen Übersetzung der Zusammenfassung, unter dem Titel „Summary" versehen werden.

Allein unveröffentlichte Aufsätze, welche zwecks Veröffentlichung anderswo nicht eingesandt wurden, kommen in Betracht.

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Chromatographic Reviews

PROGRESS IN CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS

VOLUME I

Edited by
MICHAEL LEDERER

Chromatography, electrophoresis and the related methods and techniques have undergone enormous expansion in the last years and their applications to the various problems are so numerous that it is hardly possible to obtain an up-to-date survey of the whole field. The editor has therefore taken up the idea of each year inviting various specialists to write a review on those aspects of the subject where such a survey is most needed. These reviews, which have previously appeared in the Journal of Chromatography, will be collected annually, translated into English when originally in French or German, and published in this series. They will thus serve to keep the reader informed of the latest developments in chromatography.

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PUBLISHERS' NOTE

From the very beginning, the aim of the Editorial Board and the Publishers of Clinica Chimica Acta has been to give the Journal an international character. Both the composition of the Editorial Board and the wide geographical range of the papers accepted for publication reflect this aim.

One important means of achieving the objective is clearly the establishing of closer links with the National Associations or Societies of Clinical Biochemists in different countries.

We are pleased to announce that as a first result of negotiations, an arrangement has now been made with the

Association of Clinical Biochemists (Great Britain),

Nederlandse Vereniging voor Klinische Chemie (The Netherlands),

Société suisse de chimie clinique,
Schweizerische Vereinigung für klinische Chemie (Switzerland),

whereby the Journal will appear, as from January 1960, under the sponsorship of these organizations.

It has also been decided that there will be two Editors-in-Chief, one from the United Kingdom, dealing mainly with papers from English-speaking countries, and one from continental Europe, dealing mainly with papers from continental European countries.

The Publishers are happy to announce that Prof. Dr. K. HINSBERG (Düsseldorf) has agreed to continue as continental Editor-in-Chief, and have pleasure in welcoming Dr. C. P. STEWART (Edinburgh) as his British colleague. They are also pleased to welcome Professor N. F. MACLAGAN, Dr. J. C. M. HATTINGA VERSCHURE and Dr. M. C. SANZ in their new capacity as representatives on the Editorial Board of the British, Dutch and Swiss Associations respectively.

THE SYNTHESIS OF ANTIBODIES AND PROTEINS*

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Behringwerke AG, Marburg/L. (Deutschland)

In the last few years biochemists have been very successful in the investigation and the chemical synthesis of active biological substances. This progress has extended to the secrets surrounding the protein, that vital source of biological and biocatalytical activity.

The role of amino acid sequence in protein chemistry

The work of numerous investigators has shown that the properties of proteins and their special functions as enzymes, hormones, toxins etc., depend on their chemical

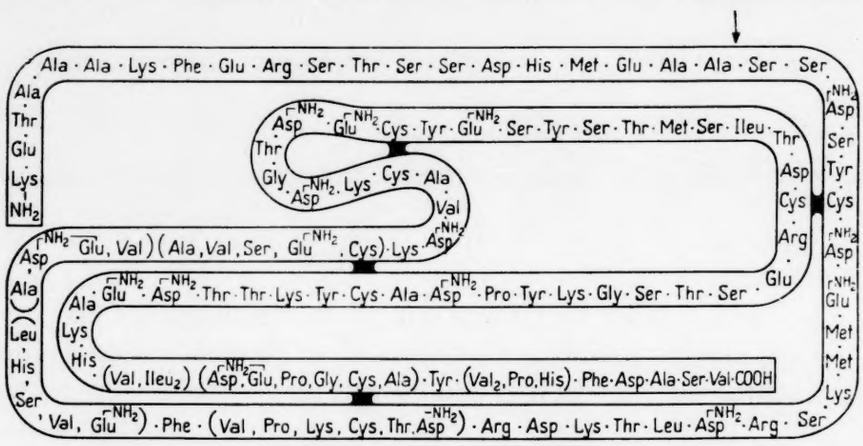

Fig. 1. The peptide chain of the ribonuclease molecule.

constitution and particularly on the sequence of the different amino acids contained in each protein. However, it has been shown that only a certain site of the protein molecule is responsible for a particular activity[1]. For example the sequence, aspartic acid–serine–glycine, was found to be an important constituent of the active range in the peptide chains of the proteolytic enzymes, α-chymotrypsin[2–5], trypsin[6–8] and thrombin[9]. The active centre of the ribonuclease molecule which consists of one peptide chain of 124 amino acids[10, 11] was discovered[12] in the area surrounding the 20th and 21st amino acid (Fig. 1) counted from the terminal free aminogroup.

Deviations in the sequence of the peptide chains were also noted in proteins like insulin[13] or cytochrome c[14–16] when prepared from different animals. In the case of serum albumins the influence of species specificity is easily detectable by determination of the end groups of the chain (Table I). The differences are especially evident in albumins produced from unrelated animals.

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* Paper presented at the 7th Colloquium on "Protides of the Biological Fluids, Bruges, May 1959.

References p. 623/626

TABLE I

END GROUP COMPOSITION OF SERUM ALBUMINS OF DIFFERENT ORIGIN

Species	*NH_2-end*	*COOH-end*
Man[17]	Asp·Ala·	·Gly·Val·Ala·Leu[19, 20]
Ox[17]	Asp·Thr·	Asp·Glu·Lys·Ser·Val·Thr·Leu·Ala[19]
Horse[18]	Asp·Thr·	Val·Ser·Leu·Ala[18]
Ass[18]	(Asp·Thr·)	Ser·(Val·Lys)·Leu·Ala[18, 20]
Mule[18]	(Asp·Thr·)	(·Leu·Ala)[18]
Rabbit[20, 21]	Asp·	Leu·Ala[20]
Sheep[20]	Asp·	(Glu·Asp·Thr)·Ser·Val·Lys·Leu·Ala[20]
Monkey[20]	Asp·	(Asp·Glu)·Ser·Lys·Val·Leu·Ala[20]
Pig[17, 20]	Asp·	

These examples demonstrate the great importance of chemical investigations of proteins. The knowledge of the chemical constitution of the peptide elements provides us with the most reliable information not only as to the specific action, but also as to the origin of a protein. This fundamental part of protein structure, called the primary

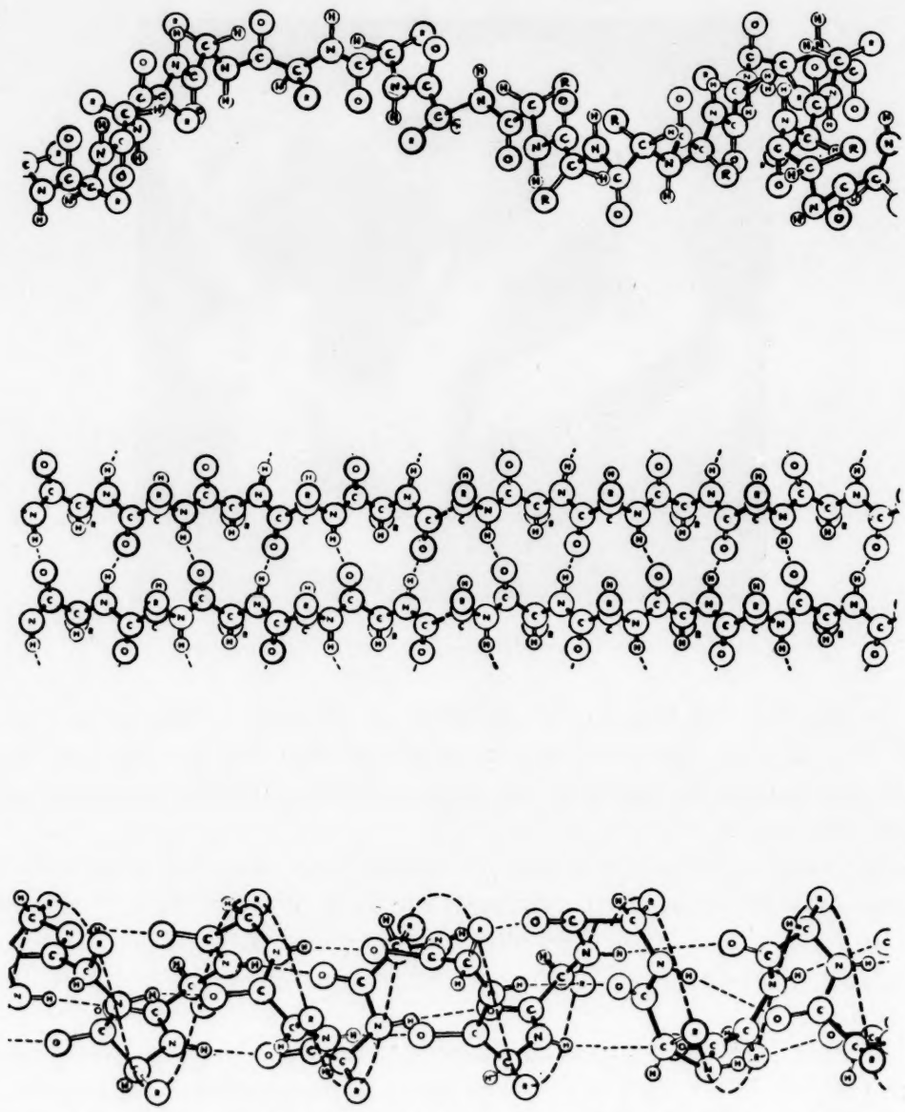

Fig. 2. Secondary structure of peptide chains[22]. α-Helix; β-configuration; arbitrary chain.

References p. 623/626

structure, is hereditary, which means that the synthesis of peptides is controlled by genes. It determines the secondary structure (Fig. 2) of the different types of folding of the peptide chains and finally the tertiary structure (Fig. 3), which represents the three-dimensional configuration of the proteins which are usually of very high molecular weight.

The determining influence of the primary structure on the properties of the whole molecule is clearly shown by the hemoglobins Hb A, Hb S and Hb C[24]. Their differences in structure and electrophoretic behaviour arise from the fact that of the total amount of 300 amino acids of Hb A one single glutamic acid group is substituted by valine or lysine in the two other hemoglobins.

The nature of antibodies

The only type of protein whose biological activity is not hereditary but acquired by antigen stimulus of exogenous or endogenous origin is the antibody. Like the enzymes, proteohormones and pharmacologically efficient proteins, the antibodies

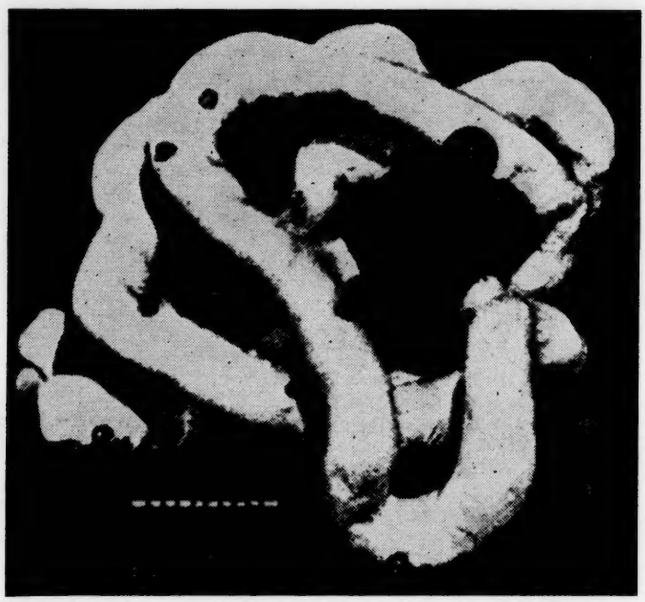

Fig. 3. Three-dimensional model of the myoglobin molecule[23].

are characterized by a high degree of specificity in their mode of reaction. Even in the early days of antitoxin discovery BEHRING found that the binding and neutralizing capacity of the diphtheria antitoxin or of the tetanus antitoxin was extended only to the homologous toxins.

According to recent investigations, the antibodies—whether derived from animals or humans—may be considered as representatives of a genetically related class of proteins. In view of their generally slow mobility and of their immunochemical behaviour (Fig. 4) it seems advisable to regard the antibodies as components of the γ-globulin system.

Most human *γ-globulin* is poor in carbohydrates and proteins with the slow sedimentation rate of $S = 7$ (mol. wt. = 156,000). In immuno-electrophoretic analysis it shows an extended precipitation band instead of the usual circular band (Fig. 4b).

References p. 623/626

Inhomogeneity is confirmed by the chemical analysis of the slower and faster moving fractions, which differ in the content of neuraminic acid[25]. This phenomenon can be explained by the immuno-chemical relationship of several protein individuals deviating from each other in electrophoretic mobility.

Another characteristic of this fraction is the occurrence of numerous antibodies (shown in Table II).

The small β_2A-fraction, isolated by the HEREMANS[26] procedure, also shows signs of inhomogeneity as regards its behaviour in immuno-electrophoresis (Fig. 4e) and the complex composition of antibodies. The carbohydrate content of β_2A-globulins is much higher than that of the main fraction mentioned above.

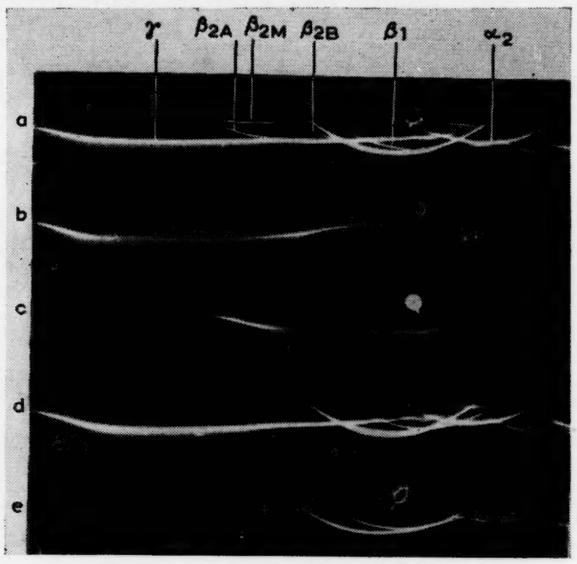

Fig. 4. Immuno-electrophoretic analysis of human γ-globulin components. (a) normal human serum; (b) normal γ-globulin components $S_{20,w} = 7$, poor in carbohydrates; (c) β_2A-globulin components $S_{20,w} = 7$, rich in carbohydrates with traces of β_2M-globulin $S_{20,w} = 18$, rich in carbohydrates; (d) normo-γ-globulinemia; (e) agammaglobulinemia. All tested with rabbit antiserum against human serum.

The macro molecular β_2M-globulin[27-30] is identical with γ_1M[31-33] ($S = 18$–20, mol. wt. = 900,000), which has not yet been isolated with a high degree of purity, and is also rich in carbohydrates.

Its electrophoretic mobility corresponds to that of the β_2A-globulin but, unlike the components with the sedimentation constant $S = 7$, the β_2M-globulin is altered by the action of sulphydryl compounds like cysteine or mercaptoethanol, the sedimentation constant being reduced by such treatment from $S = 18$–20 to $S = 7$[34-37]. This dissociation of the macro molecular component of the γ-globulin system is accompanied by a total loss of antibodies. In our experiments with γ-globulin fractions accumulated in β_2M we found a parallel increase of antibodies against polysaccharides of blood groups or bacteria (Table II). Pneumococcus-polysaccharide antibodies from horse, ox and pig[38, 39] also belong to the macro molecular type.

Finally, *properdin* belongs to the group of macro molecular antibodies. PILLEMER, BLATTBERG, NELSON and others[40-43] have shown that it has heterophilic activities (Table II) and moves in electrophoresis at the rate of the fast γ-globulins or slow β-globulins.

References p. 623/626

The occurrence of other representatives of the γ-globulin system with specific affinities is possible. We agree with GRABAR that these γ-globulins may function as transport substances primarily for metabolites in a general physiological mechanism but secondarily also for antigenic materials of endogenous or exogenous origin. Recent experiments in the field of plant hemagglutinins[45] have revealed that the protein of

TABLE II

DISTRIBUTION OF NATURALLY ACQUIRED ANTIBODIES IN THE COMPONENTS OF HUMAN γ-GLOBULIN SYSTEM

*γ-globulin components**	*γ-globulins low in carbohydrate***	*β_2A-globulin****	*β_2M-globulin*	*Properdin*
	Antibody activity§			Antibody against:
Diphtheria	+++	++	+	zymosan
Tetanus	++	+	++	levan
Typhoid H	++	+	++	dextran
Typhoid O	+	++	+++	polysaccharid
Paratyphoid B	+	+	+++	(shiga, coli a.o.)
Pertussis	+			stromata of
Poliomyelitis typ I–III	++	++	++	erythrocytes and
Isoagglutinins (anti-A a.B)		+	+++	organ cells
				phagues
				New castle virus
				protozoes
				rh-agglutin.
				cold-agglutin.
				reagins
				lues-antib.
				Forssman antib.
				hemolys. (rabbit)
Electrophoresis§§	0.5–2.1	1.2–3.6		
Sedimentation constant§§§	7.1	7	18–20	27
Hexoses	1.4	3.2	4.2	
Fucose	0.19	0.22	0.46	
Acetylhexosamine	1.1	2.9	3.0	
Acetyl neuraminic acid	0.23	1.8	2.0	

* In total γ-globulin fractions: antibodies against scarlet fever, smallpox, herpes, varicella, rubella, infect. and hematog. hepatitis and infect. mononucleosis.
** Free of β_2A- and β_2M-globulin.
*** Traces of β_2M-globulin.
§ Own results.
§§ pH = 8.6; $-u \cdot 10^{-5}$ cm^2/V$\cdot$ sec.
§§§ $S_{20,w} \cdot 10^{-13}$ cm/sec$\cdot$ dyn.

the seed of *laburnum alpinum*, which has a high binding capacity for blood group substances, also has the physicochemical characteristics of a γ-globulin. We have, therefore, to include a large group of naturally occurring proteins in our system of γ-globulins or binding globulins, and we may conclude that there is only a gradual and not a fundamental difference between the so-called normal γ-globulins and those of an acquired specificity, namely the antibodies.

The individual antibodies can naturally differ in electrophoretic mobility as well

References p. 623/626

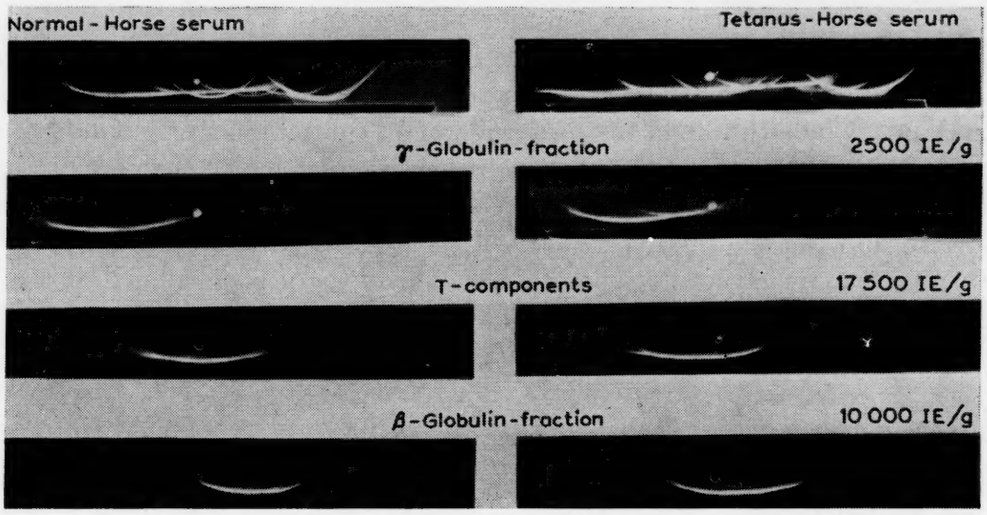

Fig. 5. Immuno-electrophoretic analysis of isolated globulin fractions of a normal and a hyperimmunised horse.

as in chemical composition [47-48]. However, no chemical difference has yet been detected between a γ-globulin with a high antibody activity and a γ-globulin of the same rate of migration with no, or only a low, antibody specificity [49].

Fig. 5 illustrates the conformity in the immuno-electrophoretic test with rabbit antiserum against horse serum, of γ-globulin fractions from a normal horse and those from a horse hyperimmunised with tetanus-toxin, both separated by zone electrophoresis. According to Table III no differences in carbohydrate content can be observed in the corresponding fractions.

The probability that the acquired immune specificity could be accompanied by a deviation—possibly only slight—in the primary structure, *i.e.* in the amino acid composition, cannot be excluded, because the complete analysis of the γ-globulin peptide chains has not yet been carried out. But in the case of rabbit γ-globulin, which consists

TABLE III

CARBOHYDRATE CONTENT OF ISOLATED GLOBULIN FRACTIONS OF A NORMAL AND A HYPERIMMUNISED HORSE

Protein	*Antitoxin units/g*	*Electrophoretic mobility**	*Hexoses*	*Acetyl hexosamine*	*Acetyl neuraminic acid*
(a) Before immunisation					
Normal-γ-globulin		1.7	1.1	1.1	0.22
Normal-T-globulin		4.4	2.1	1.9	0.89
Normal-β-globulin		5.5	3.3	2.8	1.74
(b) After immunisation					
Anti-tetanus-γ-globulin	2,500	1.9	1.1	0.9	0.25
Anti-tetanus-T-globulin	17,500	3.5	2.0	1.9	0.69
Anti-tetanus-β-globulin	10,000	4.4	2.6	2.3	1.49
Anti-diphtheria-γ-globulin	5,000	1.7	1.5	1.4	0.39
Anti-diphtheria-T-globulin	25,000	3.5	2.2	1.9	0.71

* pH = 8.6; $-u \cdot 10^{-5}$ cm^2/V · sec.

References p. 623/626

of a single peptide chain, the same pentapeptide sequence was found in a normal and in an antibody-containing γ-globulin[50]. HAUROWITZ' view, now generally accepted, is that the antibody specificity is caused merely by an abnormal mode of folding of the peptide chains, which results in the formation of a "combining site" complementarily adjusted to one of the "determinant groups" of the antigen molecule[51]. We have, therefore, to distinguish between the hereditary specificity of proteins based on the amino acid pattern and the acquired specificity of γ-globulins caused by alterations in the secondary or tertiary structure. This statement leads to the conclusion that the mechanism for the synthesis of the peptide chain of antibodies is the same as that for all the other proteins.

The formation of peptide bonds and proteins in general

The energy required for the formation of the typical peptide bonds is supplied by the following reactions:

$$\text{Amino acid} + \text{E} + \text{ATP} \rightleftharpoons \text{E} \cdot \text{AMP} \cdot \text{amino acid} + \text{PP} \quad (1)$$

$$\text{E} \cdot \text{AMP} \cdot \text{amino acid} + \text{amino acid} \rightleftharpoons \text{E} \cdot \text{AMP} + \text{peptide} \quad (2)$$

E = specific enzyme ATP = adenosine triphosphate
PP = pyrophosphate AMP = adenosine monophosphate

Generally the reaction site of this first step in protein synthesis[52] is the living cell (Fig. 6). However, the formation of peptides and even of proteins also takes place in

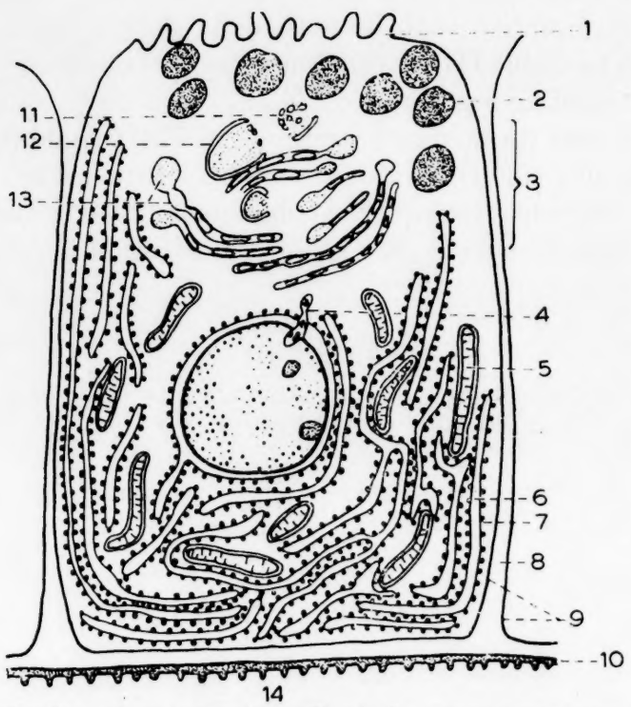

Fig. 6. Stepwise organization of an exocrine cell of the mouse pancreas (Scheme according to SJÖSTRAND[58]). 1 and 9: cell membrane (60 Å in diameter); 2: zymogen granules (store of enzymes); 3: GOLGI apparatus, consisting of double membranes with vacuoli inside; 4: breaking through of nucleolar substance (RNA); 5: mitochondrion, bordered by an outer double-edged membrane, inside with cristae (producer of energy, store of energy; dimensions $^1/_2\,\mu \times 3$ to $8\,\mu$); 6: basement cytoplasmic membranes with = 7: granula attached to one side, probable microsomes (loci of protein synthesis, dimensions $\sim$ 140 Å in diameter); 8: space between two cells; 10: basement membrane; 11–13: particles of the GOLGI apparatus, vacuoli.

References p. 623/626

tissue slices or homogenates if the following factors are present: adenosine-triphosphate, an ATP-generating system, guanosine-triphosphate or guanosine-diphosphate, microsomes and a soluble cell extract[53–57].

The soluble enzymes and ATP have been found to effect the initial carboxyl activation of amino acids[59]. The activating enzymes are probably specific for the different amino acids[60–61]. The adenyl-amino acids are extremely reactive intermediates and exist probably only in combination with the enzyme. It is not yet clear whether the so-called activated amino acids are identical with adenyl-amino acids.

The soluble extract contains a labile form of ribonucleic acid named S-RNA[62], which has a low molecular weight. This RNA-derivative activates and binds amino acids in the presence of ATP by an ester bond to ribose[63–64]. The amino acids so bound to ribonucleic acid are subsequently transferred to microsomal protein, and this transfer is dependent upon guanosine-triphosphate. The ribonucleoprotein particles

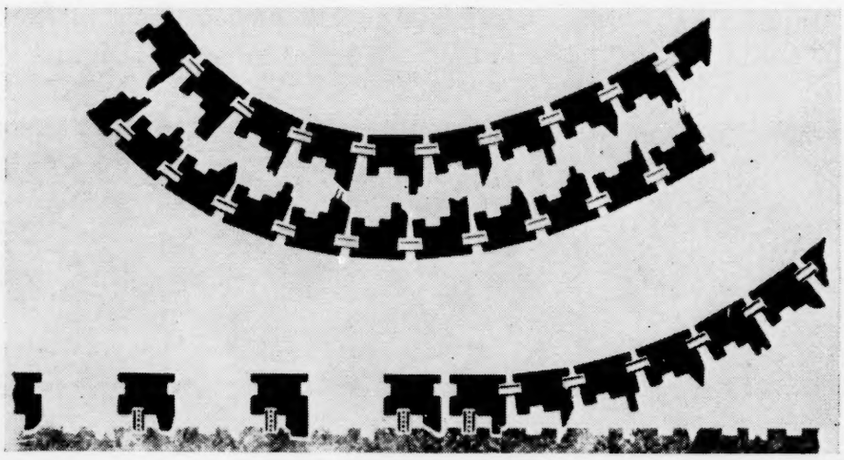

Fig. 7. Polypeptide synthesis (ZAMECNIK's scheme[68]). The amino acids are activated by ATP and bound on the phosphoric group of a RNA-matrix. Unfolding takes place after the formation of peptide bonds. The upper part demonstrates the folding and uniting of two chains.

of the microsomes appear to be the actual site of peptide condensation. It is quite possible that the amino acids also combine with mononucleotides and that these nucleotidyl–amino acid complexes react with each other to form a nucleoprotein[65]. In fact, the rate of protein synthesis is controlled by certain mixtures of nucleotides.

The role of DNA (desoxyribonucleic acid) in protein formation is not yet clear. As no correlation could be found in the biosynthesis of these two macromolecules, most investigators believe that DNA acts not directly but through RNA in the reactions leading to protein formation. DNA may be considered primarily as a template, as a more or less rigid organizer, which orientates the amino acids during the process of protein synthesis. But, as demonstrated by SCHRAMM[66], RNA can act as a template and transfer "information" on virus protein formation in the host cell. The special mechanism by which the peptide chain is unbound from the matrix and folded is not yet known[67].

Some idea may be derived from the scheme of polypeptide synthesis illustrated in Fig. 7.

A specific sequence of amino acids in the peptide chain can also be attributed to the action of a series of highly specific enzymes. The term zymosequential specificity,

References p. 623/626

however, has been coined for an assumed kind of enzymes which continually change their specificity[69]. The older enzyme theory, which explained the transfer of specificity by a successive action in the filing of amino acids in a chain, has been refuted by experiments with isotopes. Whichever theory we prefer, the template or the enzyme theory, we have reason to believe that the specificity of a protein based on a typical sequence of the amino acids in its peptide chain is genetically determined.

With regard to the antibodies, we know that they have the same amino acid composition, the same terminal amino acids and most probably also the same amino acid sequence as other γ-globulins without the special activity, therefore we must investigate the influence of genes in the different steps of antibody formation.

Origin of antibodies

One of the most important discoveries about antibodies is the observation that the liver, which is the principal organ of protein synthesis, is seldom engaged in antibody production[70-72]. If antigen is injected intravenously, most of the antibody is formed in the spleen[73] or in the bone-marrow. Local injections lead to antibody for-

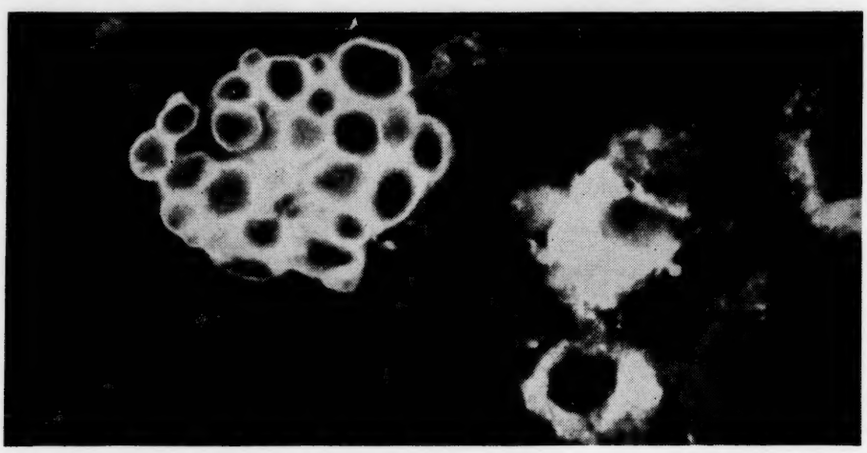

Fig. 8. Plasma cells stained by the COONS' technique. Mesenterial lymph node with plasma cells containing antibody.

mation in the regional lymph nodes[74, 75]. It is possible that antibodies synthesized by different cells have a different electrophoretic mobility and different amino acid end groups[76, 77].

FAGRAEUS[78-82] first discovered a relationship between antibody production and the appearance of plasma cells in the spleen and lymph nodes of immunised animals. She also observed an increase particularly of immature plasma cells in tissue cultures of extirpated spleen tissue from rabbits sensitized with typhoid vaccine. The origin of the plasma cells is not yet clear. Some investigators[78-83] suggest that, like the plasma cells produced in the bone-marrow of patients suffering from myelomatosis, they stem from reticulum cells, while others[83-87] regard them as transformed lymphocytes.

The histochemical identification of antibody-containing plasma cells has been much facilitated by COONS'[88-92] tracer method, using fluorescein–isocyanate, which is easily bound by proteins. As a result of an immune reaction the stained antibody is fixed by those cells containing the homologous antigen or by the cells engaged in antibody formation, if a layer of the specific antigen is artificially deposited on their sur-

References p. 623/626

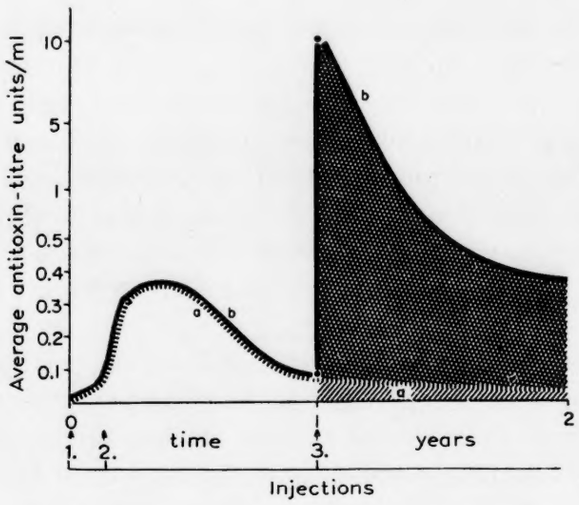

Fig. 9. The "booster" effect[95]. Increased tetanus antitoxin production after a "booster" dose of tetanus toxoid given one year after a basic immunisation by two antigen doses.

face. Fig. 8 demonstrates the plasma cells containing antibody in a lymph node[93] luminous in the fluorescence microscope.

COONS *et al.*[94] succeeded in demonstrating that many more plasma cells are formed after a second or a third application of an antigen than after a single one. Thus a well-known reaction, the so-called "booster" effect, which is characterized by an enhanced antibody production following repeated antigen applications (Fig. 9), can now be explained by a stress-like stimulation of plasma cells.

Another very important observation concerning the dependence of antibody formation on the presence of plasma cells was made by GOOD[96], who found that patients with agammaglobulinemia were unable to produce plasma cells as well as antibodies. Table IV gives an idea of the close relation of the synthesis of agglutinins against typhoid H-antigen to normal and deficient plasma cell production.

The newborn infant is unable to produce plasma cells[97, 98], but as soon as he

TABLE IV

PRODUCTION OF PLASMA CELLS AND ANTIBODIES BY NORMAL CHILDREN AND PATIENTS SUFFERING FROM AGAMMAGLOBULINEMIA

	Age	*Level of typhoid H-agglutinins in the serum after 2 antigen applications*	*Increase in plasma cells*
Normal child			
R. L.	10	20,480	+++
W. O.	7	10,240	++++
R. A.	7	10,240	+++
K. O.	7	20,480	++++
Patients with agammaglobulinemia			
E. S.	7	0	—
W. A.	7	0	—
L. L.	30	0	—
F. H.	58	0	—

References p. 623/626

starts to produce γ-globulin with antibody activity, plasma cells can be observed. The rate of γ-globulin synthesis in the first 8 months of life is illustrated in Fig. 10, which is taken from a publication by GITLIN AND JANEWAY[99, 100]. This synthesis begins only when the transferred maternal γ-globulin has disappeared. The newborn infant is also unable to synthesize the other components of the γ-globulin system, such as the β_2A- and β_2M-globulins. In general their synthesis does not begin until the third month[101–106].

Finally the properdin, which is considered to be a representative of heterophilic antibodies, belongs to the group of immune globulins which are deficient in the early months of life[107].

The question whether antigens are responsible for the production of antibodies in newborn infants induced LAURELL[108] and KENT[109] and their co-workers to study the synthesis of γ-globulins in the newborn rat and chicken bred under sterile conditions. These important investigations showed (as we learned from LAURELL) a remarkable diminution of γ-globulin production, demonstrating the importance of exogenous antigens as stimuli.

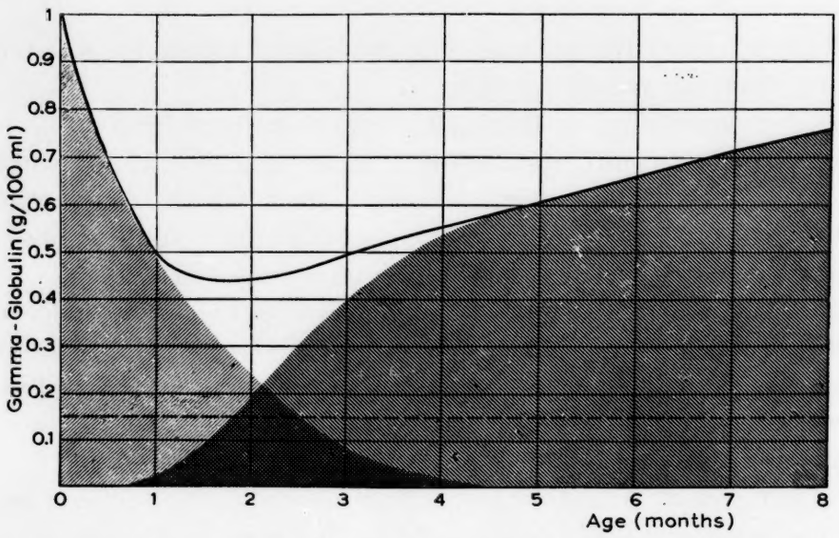

Fig. 10. The γ-globulin level in the first months of life. Left side: γ-globulin transferred from the mother; Right side: Development of the child's own γ-globulin production.

All these observations lead to the conclusion that the components of the γ-globulin system, which is the only class of protein equipped with antibody activity, are formed by special cells which are not engaged in the general mechanism of protein formation and which obey special laws of synthesis.

Antibody formation

The first attempts to discover the special steps of antibody formation concentrated on the investigation of the fate of parenterally applied antigens. HAUROWITZ[110–113] injected bovine albumin, bovine γ-globulin or ovalbumin coupled with organic components containing radio-active carbon and sulphur into rabbits and found that parts of these marked antigens were piling up in the cytoplasma of spleen and liver cells. The microsomes and later the mitochondria proved to be particularly rich in radio-activity. Fig. 11 shows that parts of a single dose of an antigen containing a maximum of 30 mg ^{35}S were still demonstrable in the mitochondria fraction three months later. This is borne out by our own experiments in which we found the im-

References p. 623/626

munological test for human albumin positive in the liver extract of mice immunised by a single intravenous dose of the human albumin antigen given 3 months earlier.

The rate of disappearance of the cell-bound antigen was much lower than that of extracellular antigen. Though the portion of the radio-active antigen taken up in 24 h in the cytoplasma of rabbit liver cells was not more than 15 μg, corresponding to 2000 antigen molecules for each liver cell, 10% of this small quantity persisted for 300 days. After 3000 days the calculated number of antigen molecules in every cell would be 20. CAMPBELL[114, 115] confirmed this long persistence in experiments with bovine albumin, which he tested by the SCHULTZ-DALE-method in rabbit liver cells; 1/100,000 part of the dose was still detectable after 3 years. INGRAHAM[116] and MCMASTER[117–119] confirmed

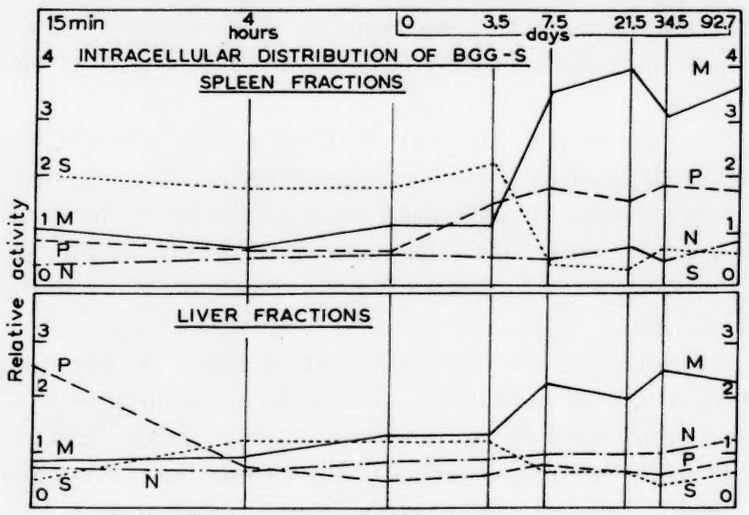

Fig. 11. Distribution of [^{35}S]-sulphanilic acid-γ-globulin (ox) in the spleen and liver cells of rabbits[112,113]. (13–30 mg antigen/kg rabbit, single dose).
..... nuclear fraction; ——— mitochondria fraction; – – – microsomal fraction; —·—·— soluble fraction.

the long retention of protein antigens in tissue cells. Moreover, according to the experiments of FELTON[120–122] with pneumococcus polysaccharides, carbohydrate antigens persist for more than 50 weeks in the spleen of mice. These observations lead us to suppose that the intracellular bound antigens play a direct part in antibody formation, and given specific information on regional protein synthesis as long as they persist despite gradual depletion due to cell division. HEIDELBERGER[123, 124] injected 50 μg of type II pneumococcus polysaccharide and by frequent estimations by the precipitation method he found that this small quantity stimulated 50 g of specific antibody during a period of 4 years. But immunologists know from many years of experience that much smaller doses of microbiological antigens contained in vaccine are sufficient to stimulate the production of protecting doses of antibodies[125].

One of the limiting factors of antibody production seems to be the restricted number of cells suitable for this purpose. This suggestion is supported by the results of the experiments of EHRICH[126], LEDERBERG[127, 128] and WHITE[129], who found with different techniques that one cell is capable of producing only one type of antibody. This accords with the classical investigations of LANDSTEINER[130], who used artificial antigens and showed that the production of antibodies with more than a single specificity is not possible if the determinant groups of the antigen are distributed on different

References p. 623/626

sites in the molecule. Antigens with two (or even more) determinant groups on the same site of the molecule sometimes cause the production of heterologous antibodies.

We are unable to explain these influences of antigen constitution on the antibody production. We may assume that not the whole antigen molecule but fragments of it act as a matrix in developing the antibody specificity. However, it is also conceivable that a nucleic acid or a derivative of it plays a part in transformation reactions by which parts of the antigen molecule, especially those foreign to the medium of protein synthesis, are activated for a specific induction of the protein synthesis. The second assumption would be supported by the observations of FAGRAEUS[78-82], EHRICH[131], HARRIS[132] and MAKINODAN[138], who found that the optimum of antibody formation in a cell was regularly accompanied by an optimum of ribonucleic acid formation. CAMPBELL[114, 115] even succeeded in isolating an antigen–ribonucleic acid compound from a liver extract of a rabbit which had been immunised with bovine albumin some time earlier.

As regards the next step in antibody synthesis these observations lead us to the further assumption that parts of the antigen molecules bearing one or a few neighbouring determinant groups are activated intracellularly by ribonucleic acid. The resulting products must be resistant to the enzymes of the cell metabolism, because they persist in the cytoplasm for a long time during which they are probably transferred from mother to daughter cells. The specific influence of these stable antigen compounds on the protein synthesis is reminiscent of genes. As the antigen compounds are not hereditary, it seems permissible to call them pseudogenes.

The synthesis of intracellular active antigen complexes has not yet been examined but it is different from the process of antibody production. We learn from extensive experimental work on bone transplantation that transplanted spleen or lymph node cells from immunised animals produce antibodies even after exposure of the receptor animal to X-rays. The same treatment suppresses any antibody formation in the first phase following the antigen application[134-138]. This period of antigen introduction or transduction in the special cells of antibody synthesis, which is strongly influenced by the action of granulocytes[130-143] and eosinophilic leucocytes[144-147], is also sensitive to cortisone or its derivatives[148, 149]. A further remarkable difference exists between the radio-resistant second phase and the radio-sensitive first phase of antibody production in so far as the first phase takes much longer than the second[150-152]. STAVITSKY[153] and ASKONAS[154] demonstrated that [^{35}S] methionine is quickly incorporated into the antibody protein produced by the homotransplantate of sensitized cells. They produce antibodies autonomically even *in vitro*[155-163]. The antibodies formed in the isolated second phase have the species specificity of the sensitized donor animal. This remarkable fact has been experimentally proved by GRABAR[164].

An important inhibitor of antibody production is the unbound antigen present in the blood circulation[114-115]. This phenomenon was named "immune paralysis" by FELTON[120-122] and is caused by the formation of antigen–antibody compounds which are easily eliminated and lead to the disappearance of every newly produced antibody.

A total inhibition of a specific antibody production for the whole period of life is possible by the prenatal or postnatal application of an antigen. The mechanism of such a specific blocking of antibody formation which is responsible for the so-called "immune tolerance"[165-168] is still unknown.

The word "unknown" occurs rather frequently in this short report. This is char-

References p. 623/626

acteristic of the present situation in the field of protein synthesis as well as in that of antibody formation. However, this fact need not perhaps depress us unduly, and may even be regarded in an optimistic light by those who fear that we may have nothing left to discuss at our next meeting.

SUMMARY

It is demonstrated that proteins with antibody activity can be regarded as a homogeneous, immunochemically related system of components, despite their differences in electrophoretic behaviour and chemical composition. Other proteins with biological activity owe their specificity to typical sequences of amino acids in their peptide chains. In the case of antibodies such a typical chemical constitution to which the specificity could be attributed has not been found as yet, and, therefore, at present the view is held that the specificity is due to alterations in the secondary or tertiary structure, resulting in the formation of a combining site complementarily adjusted to determinant areas of the antigen molecule. Antibodies are produced by a special type of cell of the spleen or the bone marrow (after i.v. injection) or by the lymph node cells (after local injection). There is no fundamental difference between the production of antibodies and that of other proteins. Like other proteins, the antibodies are provided with the characteristics of the species specificity, determined by the chief valencies of the peptide chain. The immunological specificity characteristics are, however, evidently not controlled by genes. Two phases of antibody formation are further elucidated.

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QUANTITATION OF PLASMA PROTEINS ON CELLULOSE ACETATE STRIPS*

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INTRODUCTION

Recently we have been considering to what extent the organisation and results of our routine clinical laboratory could be improved by introducing some electrophoretic method as an alternative to the more frequently used methods for plasma or serum protein fractionation.

Preliminary trials with the method of KOHN[1], using cellulose acetate as supporting medium, showed some features that appeared attractive enough to merit further investigation. The characteristics of the migration patterns achieved by this method indicated that the criteria of a reliable routine method, that is—accuracy, reproducibility, a well-defined normal range value, ease and speed—may well be fulfilled.

Two major difficulties presented themselves: the relatively high cost of the cellulose acetate strips, and the lack of information on the quantitative evaluation of results obtained by methods using cellulose acetate.

The first obstacle we overcame partially by adapting the modified method of KOHN[2] which uses small (2.5 × 10 cm) cellulose acetate strips.

A method for assessing quantitative results on these small strips was then worked out and tested as a means of overcoming the second difficulty.

METHODS

The separation runs were done in a tank of our own design which consisted of 2 basic parts, the trough assembly (T) and the strip chamber. The construction is shown in Figs. 1–1 and 1–2.

The strip chamber has a very small internal volume. For a capacity of 6 strips, the external measurements are: length 20 cm, width 16 cm and depth 1.25 cm; this gives a vol. of 67 cm^3/strip. The side walls of the chamber are made from perspex; the floor and roof are thin polythene sheeting (G) stretched on a perspex frame and backed by a pad of plastic foam (H). This arrangement provides efficient heat insulation and prevents excessive water condensation on the walls of the tank. The airtight fitting of the lid onto the chamber is assured by the interposition of PVC plastic tubing (J) between the lid and the chamber walls, the lid being clamped down by wing nuts (L). The access of buffer solution to the strips is facilitated by filter paper wicks (R) dipping into the buffer reservoir (Q) in between the baffles (X). A sufficiently high level of buffer in the trough (Q) assures closing off the strip chamber space from the

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References p. 638

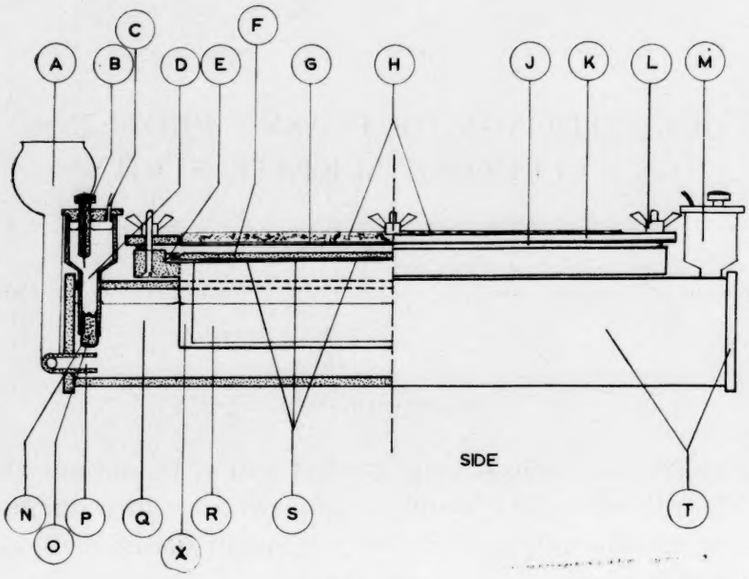

Fig. 1–1

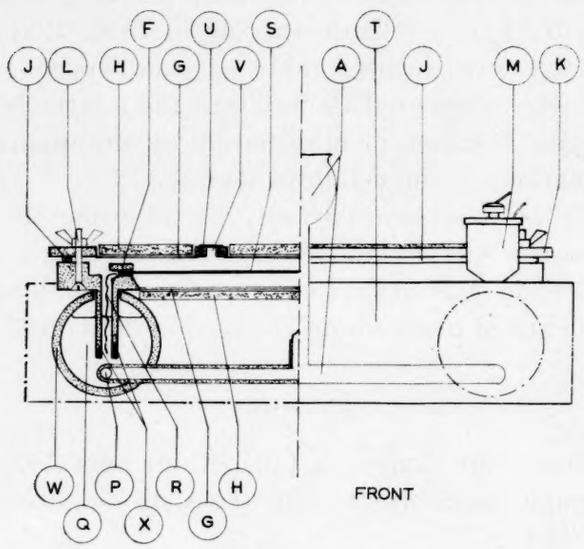

Fig. 1–2

Fig. 1. Electrophoresis tank design: side and front view.

1, 2. A: Thistle funnel of buffer filling system.
B: Platinum electrode.
C: Vent tube.
D: Buffer filled electrode vessel space.
E: Peg holding the bar for fixing strips.
F: Perspex bar fixing strips in position.
G: Polythene film cover.
H: Plastic foam insulation.
J: PVC tubing washer.
K: Tank lid.
L: Wing nut.
M: Electrode vessel.
N: Cellophane membrane.
O: Cellulose pulp plug.
P: Buffer filling and equalising system.
Q: Trough filled with buffer.
R: Filter paper extension of strips.
S: Cellulose acetate strips.
T: Trough assembly.
U: Plastic strip cover of application slit.
V: Application slit.
W: Trough cross section.
X: Perspex baffle of the chamber.

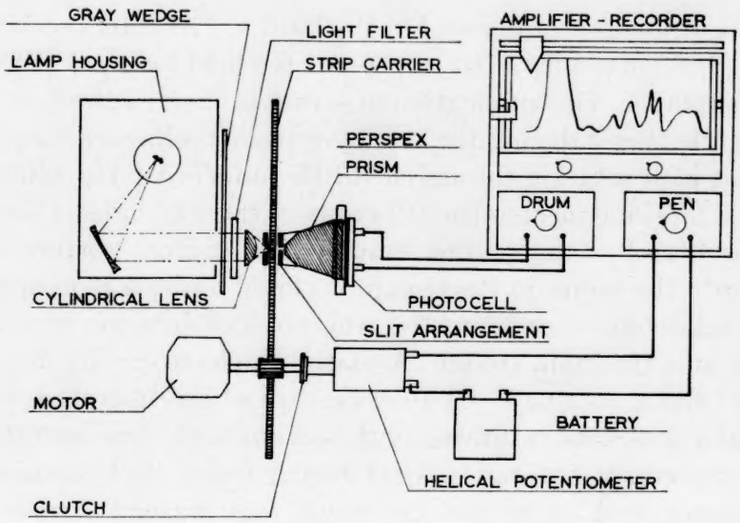

Fig. 1–3

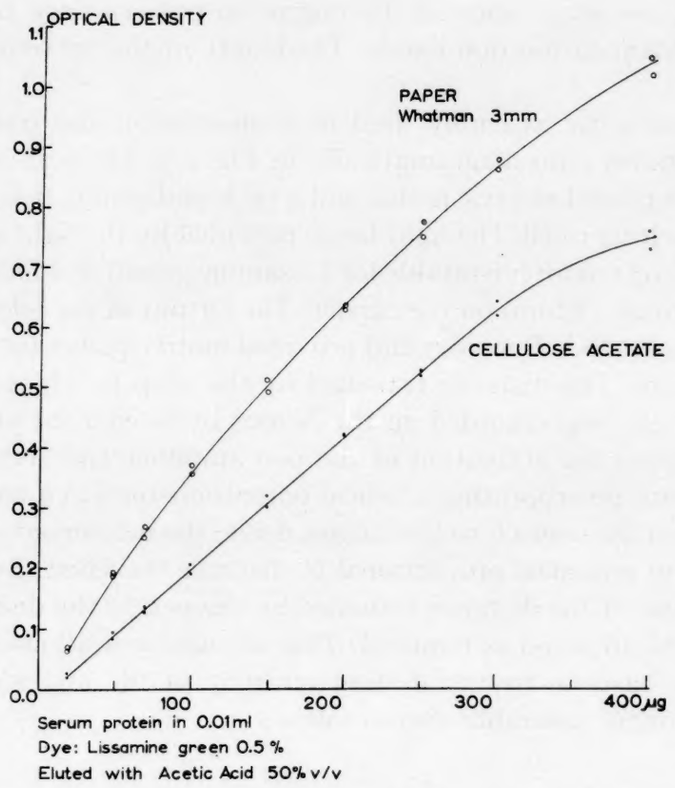

Fig. 1–4

Fig. 1. Electrophoresis tank design.

3. Scanner-Recorder assembly. Semidiagrammatic representation.

4. Relation of dye uptake to protein quantity on cellulose acetate and paper.

external air since it forms a buffer seal in the baffles. The cellulose acetate strips for electrophoresis (S) are fixed by a bar of perspex (F) held by a peg (E) projecting from the wall of the chamber. The application of serum is effected through a narrow slit in the lid (V) which is closed during the run by a plastic adhesive strip (U). The buffer troughs are filled with solution through a thistle funnel (A). The filling assembly also serves as a buffer levelling mechanism (P) between the 2 troughs. There is an electrode vessel (M) at each end of each buffer trough. The narrow portion of the electrode vessel dipping into the buffer in the trough is closed off by a plug of pulped cellulose (O) wrapped in cellophane membrane (N); this reduces diffusion of decomposed buffer from the vessel into the main trough. A platinum electrode (B) dips into the buffer (D) in the vessel and a vent tupe (C) allows escape of gas liberated on the electrodes.

Six strips at a time were saturated with sodium barbitone–acetate buffer pH 8.6[3] and run for 90–100 min at 130–140 V. After dyeing with 0.5% Lissamine green (Gurr) in 6% salicylsulphonic acid for 20 min the strips were washed several times with 5% acetic acid. The washed strips were dried between filter paper sheets and when dry were made transparent by immersion in commercial "White oil".

Thus treated, the strips showed the migration pattern on a transparent background, with well-defined fraction bands. The length of the patterns varied between 6 and 8 cm.

The scanner-recorder assembly used in evaluation of the transparent electrophoretograms is shown semi-diagramatically in Fig. 1.3. The scanner part has a strip carrier moved by a geared electric motor and a rack-and-pinion drive between a light source and a photoelectric cell. The light beam provided by the light source was passed through an Ilford 204 red filter (suitable for Lissamine green) and focused so as to form an image of a slit (0.25 × 8 mm) on the carrier. The output of the selenium cell was fed to a Moseley Autograf X-Y Recorder and provided motive power for the Y axis record (movement of drum). The distance travelled by the strip *i.e.* the position of the slit image on the carrier—was recorded on the X axis by movement of the pen. Change of potential necessary for activation of the pen amplifier and servomechanism was provided by a circuit incorporating a helical potentiometer and a battery. The potentiometer was linked by a clutch to the carrier drive, the movement of the carrier thus causing a change of potential proportional to distance travelled. By means of a suitable shunt, the ratio of the distance travelled by the pen to the distance travelled by the carrier could be adjusted as required. This arrangement allows for adjustment of the length of the baseline to any desired multiple of the migration distance. The standard setting of the assembly was as follows:

Dark current —0 deflection; O.D. ∞
Scan pattern base line } 100 divisions, transmission 100%
Full deflection Y axis } O.D. 0.00
Any response on Y axis between 0–100 is expressed as transmission percentage
Any response on Y axis between ∞–0 is expressed as O.D.

The scanning time of one strip was 20 sec and the length of the scan pattern 10–14 cm.

For evaluation of the scan patterns the method relying on the area-to-weight relationship was found satisfactory. Some papers showed great variation in the weights of individual sheets and also of different parts of the same sheet. Finally a

References p. 638

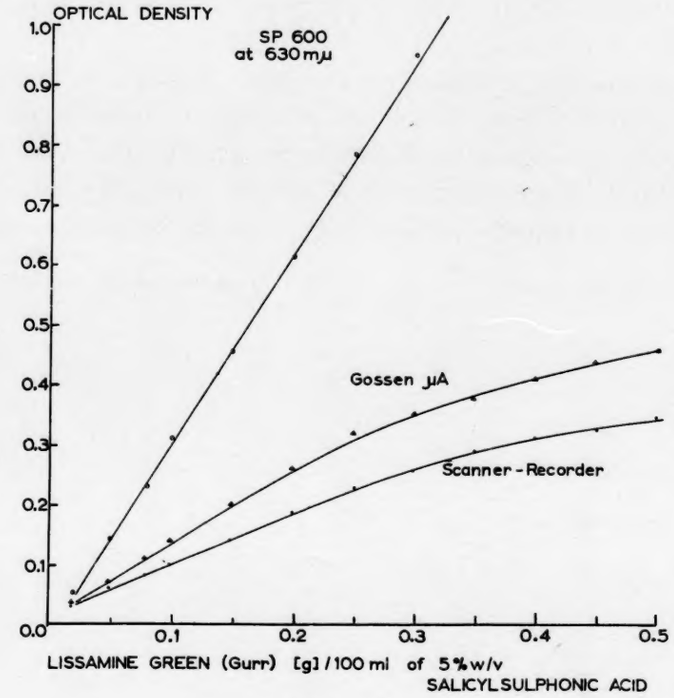

Fig. 2–1

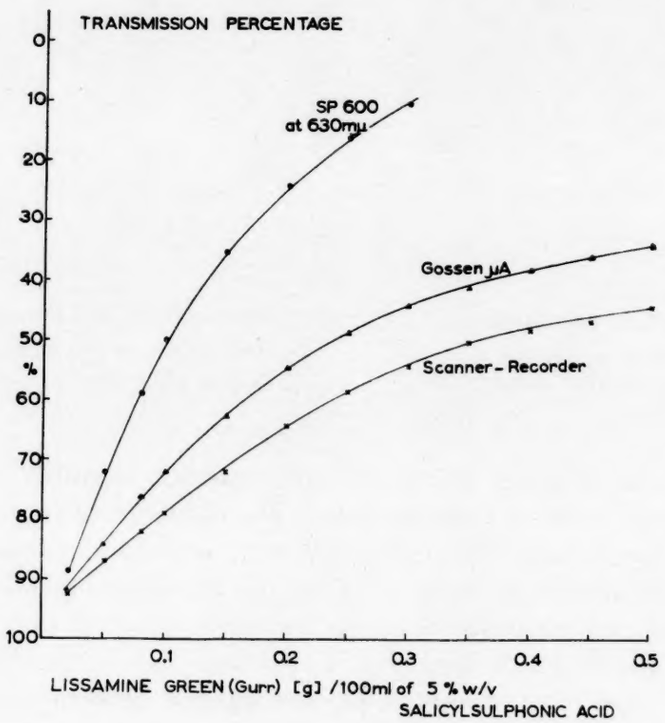

Fig. 2–2

Fig. 2. Response of apparatus to various concentrations of Lissamine green.

1. In terms of optical densities.
2. In terms of transmission percentages.

reasonably uniform brand of typewriter paper was found and this had a mean weight of 37 mg/in². (SD 0.824, V = 2.2)

The first few preliminary evaluations revealed that the values obtained for the albumin fraction were far too low. A non-linear relationship between scan patterns of standard protein spots was noted by KOHN in his paper[1] when the concentration of the protein in the standard spot exceeded a certain value. We attributed our observation tentatively to two factors: (a) lower dye uptake by protein at higher concentration; (b) non-linear response of the scanner-recorder assembly if the dye density of the scanned area was above a certain limit. The influence of protein concentration/unit area on the dye uptake was tested by applying standard volumes of protein solution, varying in concns. from 0.2 to 4.0 g/100 ml, to cellulose acetate strips and paper. After dyeing and washing the strips the spots were cut out and eluted with a standard volume of 40% acetic acid. Fig. 1–4 shows O.D. of the eluate measured at 630 mμ by SP 600 Spectrophotometer, plotted against protein concentration of the standard spots. The graph reveals that the dye uptake shows a closer approach to linearity on cellulose acetate than on paper, up to protein concn. of 3 g/100 ml, but the absolute amount of dye taken up is greater on paper.

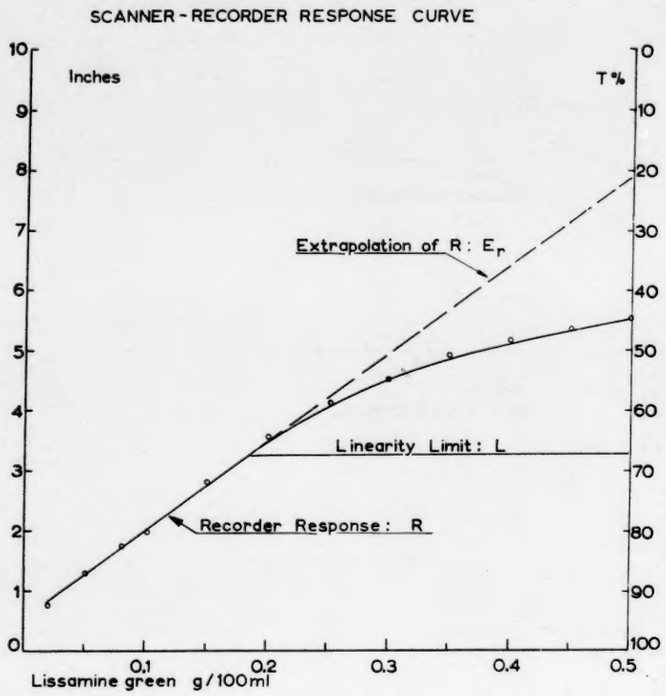

Fig. 2–3

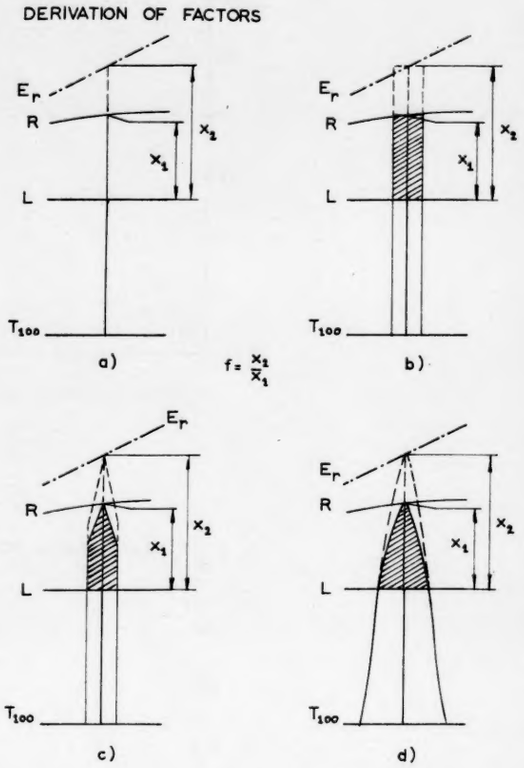

Fig. 2–4

Fig. 2. Response of apparatus to various concentrations of Lissamine green.

3. Scanner-recorder response curve. Its adaptation for calculation of correction factors.

4. Derivation of correction factors. Mode of their application for correcting various areas.

When testing the response characteristics of the scanner-recorder, the use of solutions of Lissamine green in cells of 2.0 mm optical width eliminated errors inherent in the unevenness of standard protein spots on the supporting material. The response

References p. 638

deflection of the recorder to various concentrations of the dye solutions when the cell was substituted for the strip carrier has been recorded along the Y-axis. In a parallel experiment the response was measured by a Gossen microammeter with log scale ∞ —o. The optical densities of the standard dye solutions were also measured in the same cell by SP 600. In all measurements a 6% solution of salicylsulphonic acid was used as blank.

In order to be able to compare results obtained by the different apparatus, values from the scanner-recorder registered in terms of transmission percentages ($T\%$) had to be re-calculated into optical densities values (O.D.) in terms of which the Gossen micro-ammeter and SP 600 results were recorded. Conversely, all results were re-calculated in terms of transmission percentages. The relationship of dye concentration and transmission percentage or O.D. is shown graphically in Figs. 2–1 and 2–2. The linear relationship shown when O.D., measured by SP 600, is plotted against dye concentration is not obtained with either of the 2 other instruments. The graphs representing the same relationship in terms of transmission percentages shows that the linear portions of the curves are shorter than in the previous graph. However the linear portion of the scanner response curve could be utilised for evaluation of the migration patterns in 2 ways.

Firstly, the concentration of the protein fractions could be reduced to such an extent that none would cause the dye density on the strip to be so high as to evoke scanner response within the non-linear part of the response curve. This however would involve having protein concentrations in the smaller fractions which were far too low for satisfactory measurements.

The alternative approach, which was eventually adopted, was to extrapolate the linear portion of the curve and introduce correcting factors for the non-linear part. The derivation of these factors is best illustrated by considering examples in Figs. 2–3 and 2–4. Fig. 2–3 shows response curve *(R)*, the extrapolated part of the linear section of $R:E_r$. At the point where R ceases to be linear a linearity limit line *(L)* was drawn parallel to base line (T 100%). Any response exceeding the linearity limit has to be corrected so as to extend it to the extrapolated portion and thus bring it into linear relationship with any response not exceeding the linearity limit. This is shown in Fig. 2–4a. In this instance the response at the given point exceeded L by distance x_1, which should be corrected to x_2 by a correction factor $f = x_2/x_1$. As the portion of response below L need not be corrected (*i.e.* $f = 0$), the corrected value of response would be $L + x_2$. It was essential that factors derived in this fashion and applicable for correcting a line should also be of use in correcting the areas of the scan patterns projecting above the L line. The applicability of factors derived from measurement of height (x_1) of various simple symmetrical geometrical figures was proved correct as can be seen in examples b, c and d of Fig. 2–4. These are, of course, symmetrical patterns but errors introduced by the asymmetry of some of the scan pattern areas above L proved to be of much less significance than errors inherent in the migration and separation factors. The other examples in Fig. 2–4(b, c and d) illustrate how the factors derived from the example (a) can be extended to give correction for complete areas. Analogous factors can be derived for the response curve of the Gossen microammeter or any other instrument showing similar characteristics.

To facilitate the evaluation of scan patterns, factors at various values of x_1 were calculated and plotted against the x_1 measured in mm. In Fig. 3–1 the factors are

References p. 638

plotted on an arithmetic scale, whilst in Fig. 3–2 the factors are plotted on a log scale. From graph in Fig. 3–2 a table of factors with x_1 increasing by steps of 2.0 mm was compiled (Table I). How these correction factors were applied to the scan patterns is illustrated in Fig. 3–3, 5. On the pattern, line L is drawn at the appropriate distance from baseline T 100%. To correct any fraction area of which the peak exceeds limit L, x_1 is measured in mm and a factor corresponding to it is noted. After cutting out the pattern and weighing each fraction, the fraction requiring correction is cut along line L and both parts are weighed separately. The weight of the portion above L is then multiplied by the factor. The figure thus obtained is added to the weight of the portion below L, the total representing the corrected weight of the fraction.

TABLE I

CORRECTION FACTORS

Values of x_1 increasing by steps of 2.0 mm from 0–80 mm

x_1	f	x_1	f	x_1	f	x_1	f
0	–	20	1.22	40	1.44	60	1.88
2	1.11	22	1.23	42	1.47	62	1.95
4	1.12	24	1.25	44	1.50	64	2.03
6	1.13	26	1.27	46	1.54	66	2.10
8	1.14	28	1.29	48	1.59	68	2.17
10	1.15	30	1.31	50	1.62	70	2.25
12	1.16	32	1.33	52	1.67	72	2.32
14	1.17	34	1.35	54	1.72	74	2.37
16	1.19	36	1.38	56	1.76	76	2.42
18	1.20	38	1.40	58	1.83	78	2.46
						80	2.50

TABLE II

REPRODUCIBILITY OF SCAN PATTERNS

Evaluation	*Term*[e]	*Albumin* %	*Globulin* %			
			α_1	α_2	β	γ
Series B[a]						
I	$\bar{x} \pm$ SD	53.6 ± 3.0	2.9 ± 0.6	10.8 ± 0.9	12.1 ± 1.0	20.3 ± 1.7
	v	5.5	21.7	8.6	8.4	9.2
II	$\bar{x} \pm$ SD	65 ± 2.8	2.7 ± 0.6	8.9 ± 0.8	8.2 ± 1.2	14.9 ± 2.0
	v	4.3	23.0	8.7	14.4	13.3
Series b[b]						
I[c]	$\bar{x} \pm$ SD	53.4 ± 1.6	2.9 ± 0.6	11.8 ± 0.5	11.8 ± 1.2	20.5 ± 1.2
	v	3.0	21.7	4.4	10.2	6.0
II[d]	$\bar{x} \pm$ SD	65.2 ± 2.7	2.8 ± 0.6	9.0 ± 0.8	8.0 ± 1.5	14.9 ± 1.4
	v	4.1	22.5	8.8	14.4	9.2

[a] Patterns without selection; n = 36.
[b] Patterns without blemish only; n = 20.
[c] No correction for background dye.
[d] Correction for dye in the background.

[e] Average of n strips $\bar{x} = \frac{x}{n}$

Standard deviation $SD = \frac{(\bar{x} - x)^2}{(n - 1)}$

Standard error $V = \frac{SD \times 100}{\bar{x}}$

References p. 638

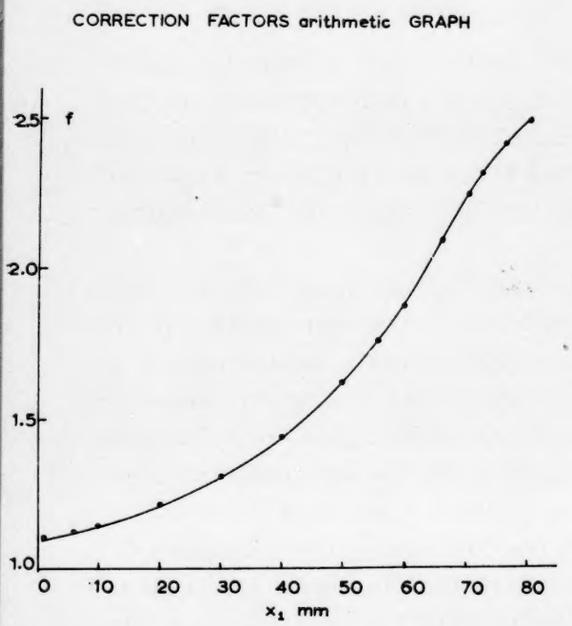

Fig. 3–1

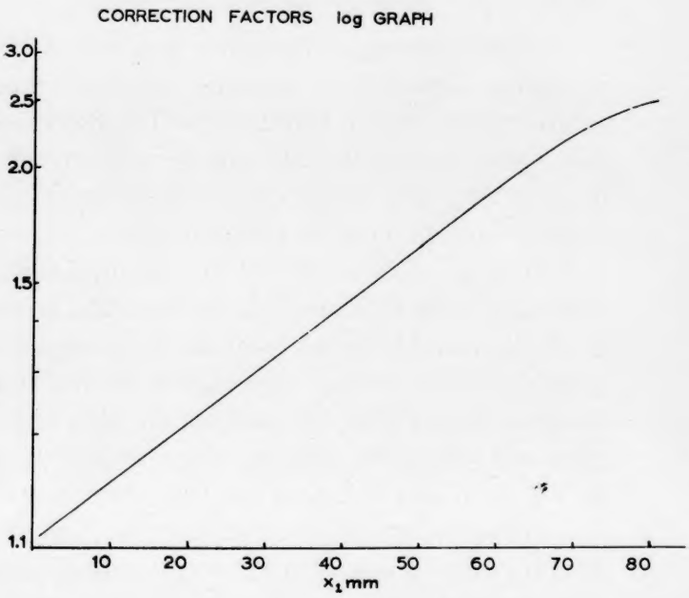

Fig. 3–2

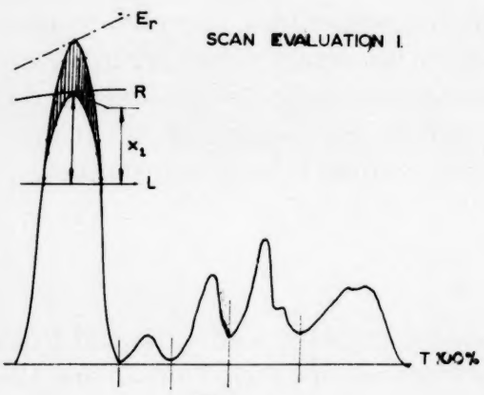

Fig. 3–3

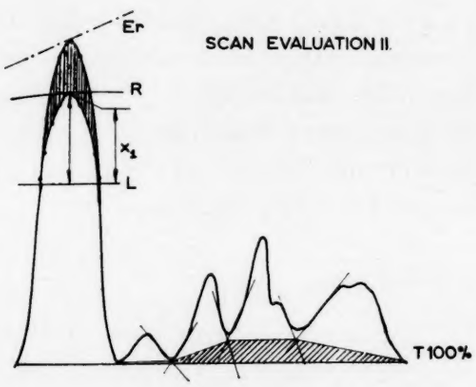

Fig. 3–4

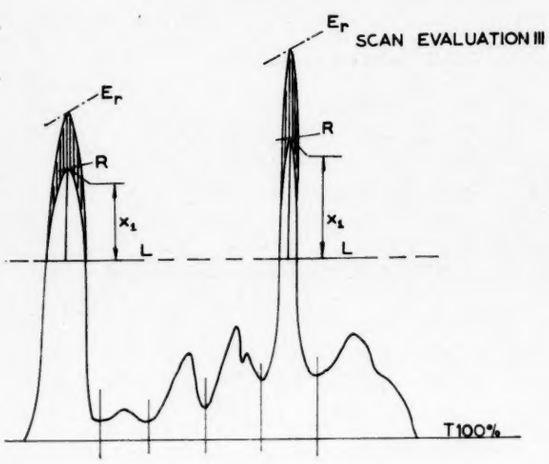

Fig. 3–5

Fig. 3. Relationship of correction factors to corresponding x_1,

1. Plotted on arithmetic:arithmetic scale.
2. Plotted on log:arithmetic scale.

Application of correction factors to scan-patterns:

3. Normal pattern.
5. Myeloma pattern.

Effects of various methods for determining fraction boundaries on scan pattern evaluation.

3. Scan evaluation I, no compensation for background dye.
4. Scan evaluation II, attempted correction for dye bound by denatured protein.

Reproducibility of the method

To test the reproducibility, 6×6 runs were done on the same serum, the amount of serum applied to the strips varying between 1.8 and 5.3 μl. Evaluation of these strips was carried out in 2 ways. The data from each evaluation type was subdivided into series designated (B) and (b) respectively. Series B consisted of all 36 strips with no selection whatever; series b excluded any of the 36 strips with some pattern distortions—leaving 20 strips in all.

In evaluation I the fraction boundaries on the scan pattern were determined by dropping perpendiculars to the baseline at the trough points between fraction peaks. In evaluation II the limbs of the fraction peaks were extrapolated downwards and the points of intersection were taken as fraction boundaries and also as the corrected baseline which compensated for the dye bound to the denatured protein in the background of the strip pattern. The effect of these procedures on the scan patterns is seen in Fig. 3–3, 4. The results of the reproducibility evaluation is given in Table II.

Albumin and α_1 globulin were least affected by the correction for background dye. The fractions most affected were β- and γ-globulins and therefore these fractions not only showed great reduction in percentage but also incurred the greatest error when an attempt at correction was made. Thus, the coefficient of variation increased after correction in Series B from 8.4 to 14.4 for the β-globulin and from 9.2 to 13.3 in the γ fraction. In Series b the rise though not so marked was still considerable: 10.2 to 14.4 for β and 6.0 to 9.2 for γ fraction. Surprisingly, errors that were expected to arise from distortion of the migration patterns are of lesser importance than errors introduced by correcting for the background dye as in evaluation II. The change in the proportions of fractions relative to each other caused by the corrections in evaluation II was to be expected and as a set of normal values would have to be established, was of little importance in itself.

Normal range values

In 10 consecutive runs, of 6 strips each, migration patterns were obtained from 60 sera supplied by the Blood Transfusion Service from routine collection among the normal donors in the Edinburgh area. The evaluation was that used in evaluation I, without selection, and in Table III the results are compared with those obtained some

TABLE III

NORMAL VALUES AND RANGE OF SCAN PATTERNS

Material	*Term*[c]	*Albumin* %	*Globulin* %			
			α_1	α_2	β	γ
Cellulose acetate[a]	$\bar{x} \pm$ SD	59.8 ± 4.14	2.4 ± 0.47	10.0 ± 1.86	12.0 ± 2.00	15.7 ± 3.10
	v	6.8	19.5	18.4	16.5	19.7
Paper[b]	$\bar{x} \pm$ SD	65 ± 4.3	7.7 ± 1.3	5.8 ± 1.4	9.3 ± 2.2	15.4 ± 3.0
	v	6.6	36	24	24	19.4
Range	$\bar{x} \pm 2$SD					
Cellulose acetate[a]		51.6 – 68.1	1.4 – 3.3	6.3 – 13.7	8.0 – 16.0	9.5 – 21.9
Paper[b]		56.4 – 73.6	1.3 – 7.7	3.0 – 8.6	4.9 – 13.7	9.4 – 21.4

[a] 60 strips evaluated. [b] 34 strips evaluated. [c] As in Table II.

References p. 638

time ago in the same laboratories by Dr. OWEN[4] using paper electrophoresis followed by reflectodensitometry.

Whilst the normal value for the albumin fraction is slightly lower on cellulose acetate than on paper; it is considerably lower for α_1; α_2- and β-globulin fractions on the other hand are higher when evaluated on cellulose acetate than on paper. The ranges are narrower for cellulose acetate with coefficients of variation much lower for α_1-, α_2-, β-globulin fraction on cellulose acetate and very slightly lower for albumin and γ-globulin fractions on paper.

TABLE IV

ALBUMIN GLOBULIN RATIOS – REPRODUCIBILITY ASSESSMENT

Term[a]	*Albumin %*	*Total globulin %*	*A/G Ratio*
Elution[b]			
$\bar{x} \pm$ SD	54.9 ± 1.3	45.1 ± 1.3	1.217 ± 0.063
v	2.4	2.9	5.2
Scanning[c]			
$\bar{x} \pm$ SD	53.6 ± 3.0	46.4 ± 3.0	1.157 ± 0.113
v	5.5	6.5	9.8

[a] n = 12. [b] As in Table II. [c] n = 36.

TABLE V

NORMAL VALUES AND RANGE ALBUMIN–GLOBULIN RATIOS

Comparison of (a) normal values determined by short run separation followed by elution (b) normal values determined by long run followed by scanning.

Terms	*Albumin %*	*Total globulin %*	*A/G Ratio*
Elution			
$\bar{x} \pm$ SD	63.5 ± 4.8	36.5 ± 4.8	1.740 ± 0.367
Range $\bar{x} \pm$ 2SD	53.9–73.1	46.1–26.9	1.168–2.320
Scanning			
$\bar{x} \pm$ SD	59.8 ± 4.14	40.2 ± 4.14	1.448 ± 0.207
Range $\bar{x} \pm$ 2SD	51.6 – 68.1	48.4 – 31.9	1.068 – 2.135

A rapid method for determining A/G ratios

The distinct separation between albumin and α_1-globulin that is a constant feature of patterns on cellulose acetate suggested the possibility of using short, rapid runs followed by elution of albumin and total globulin fractions as a rapid method for determining albumin–globulin ratios. For this purpose the running time was cut to 40–50 min at 150 V. The volume of serum applied was kept under 3.0 μl to prevent undue crowding of fractions. The stained and washed strips were cut along the albumin–globulin boundary. The 2 sections of the pattern were then eluted in a standard volume of 40% acetic acid. The elution was aided by placing the test tubes containing the acid and the strip section in a simmering waterbath for 4 min. After cooling, any precipitate that formed was centrifuged down and the O.D. of the eluates were deter-

References p. 638

mined by SP 600 at 630 mμ. From the percentage figures for albumin and globulin the A/G ratios were calculated. Evaluation of 12 runs on the same serum provided the figures for reproducibility test and are presented in Table IV.

The range of normal values was obtained from analysis of 30 sera supplied again by normal blood donors. Table V shows the results. The figures in Tables IV and V indicate that the reproducibility of this method is somewhat better than the method using a longer run followed by scanning and the range of normal values for albumin is higher with the short method, probably because the amount of denatured albumin remaining in the globulin fraction is smaller.

SUMMARY

To establish a rapid analytical procedure for serum proteins adequate for routine clinical work, protein fractions separated by electrophoresis on cellulose acetate strip have been evaluated. After a "run" of 1.5 h the strips were stained with Lissamine green, cleared in White oil and put through a transmission scanner coupled to an automatic X-Y recorder. Correction factors, which must depend upon the characteristics of the instruments used, were applied to the readings corresponding to serum fractions with O.D. above an empirically established level and the method of calculating these is described. Using these factors the range of values for normal sera was found and the reproducibility of the method was determined. Comparison was made with a very rapid method for determining the albumin: total globulin ratio for serum, using 45-min electrophoretic separation followed by the elution of the appropriate stained fractions from the cellulose acetate strip.

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IMMUNOCHEMICAL STUDIES ON PROTEIN PATHOLOGY*
THE IMMUNOGLOBULIN CONCEPT

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Since the advent of immuno-electrophoresis[1] it is possible to make a much more detailed study of the serum protein pattern than could be done by classical physical or chemical methods, such as conventional electrophoresis and salt precipitation. With the use of appropriate precipitating antisera, immuno-electrophoresis of normal serum shows the existence of 26 easily discernable protein components, and with the aid of suitably absorbed antisera it is possible to distinguish an additional number of precipitation lines, which brings the total of visualized components up to at least thirty.

The present review is concerned mainly with the proteins constituting the classical γ peak of conventional electrophoresis, and with those which can be identified in the depression lying between this peak and the β area.

The latter zone has been given many different designations, and names such as "β_2-", "γ_1-", "T-", "M-", "ζ-" and "X-" have been proposed. In the present paper the name "β_2-region" is used. It should be stressed that no correspondence with the "β_2-region" of LAURELL *et al.*[2] is suggested. The latter name is a designation for the slower component of the β peak of paper electrophoresis, when this peak is split by the addition of calcium ions to the buffer, and at least one of its components, the β-lipoprotein, is entirely absent from the agar-electrophoretic β_2 area.

A REVIEW OF THE COMPONENTS OF THE β_2-γ AREA

Under ideal circumstances five protein precipitation lines, possibly six, can be observed in the area under study. These are (Fig. 1): (a) γ-globulin, (b) β_{2A}-globulin, (c) β_{2M}-globulin, (d) β_{2B}-globulin, (e) β_{2X}-globulin, (f) γ_X-globulin.

Immuno-electrophoretic data

γ-Globulin: The precipitation line of γ-globulin has already been described in many publications, and its principal feature, the fact that it can be seen to extend far into the beta and even into the slow alpha regions, was mentioned in the very first publication of the immuno-electrophoretic method[1]. With few exceptions, all writers seem to agree that this indicates the existence of a population of antigenically similar, but electrophoretically heterogeneous, proteins which all deserve to be called "γ-globulin". In the latter sense the word has lost its electrophoretic meaning to the profit of a more immunologically minded conception.

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* Paper presented at the 7th Colloquium on "Protides of the Biological Fluids", Bruges, May 1959.

** Director: Prof. P. LAMBIN.

References p. 646

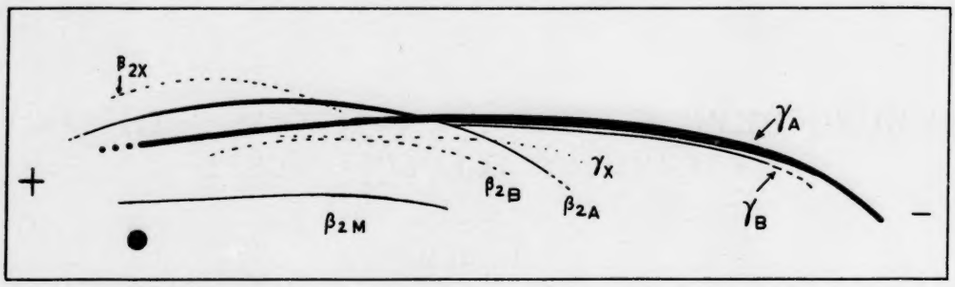

Fig. 1. Schematic representation of the β_2 and γ proteins. γ (split into A and B), β_{2A} and β_{2M} are the main components. β_{2B} and β_{2X} are trace components. γ_X is an inconstant feature seen only in pathological conditions.

A fact apparently not mentioned in the literature is that the γ-bow can regularly be seen to be split into two closely parallel precipitation lines, of which the outer one, which we may tentatively term "γ_A", is by far the more important and extends cathodically much farther than the inner one, which may be named" γ_B". These features are discussed in more detail in a later section of this paper.

β_{2A}-globulin: The first immuno-electrophoretic tracings of human serum obtained by GRABAR AND WILLIAMS[1] already showed the presence in the β_2 region of a precipitation line not identifiable with any known protein. It was first called "β_2-globulin"[1], or "β_X"[3], which names were replaced by "β_{2A}-globulin" when other components of the same area became known[4]. The β_{2A}-globulin was finally isolated from normal serum by a zinc fractionation method[5].

β_{2M}-globulin: The SCHEIDEGGER method of immuno-electrophoresis[3] allowed the discovery of another precipitation line in the β_2 region. This line was initially thought to correspond to a cryoglobulin and was called "β_{2C}"[6], but it was soon found to be produced by a protein of high molecular weight, and was called "γ_1-globulin" by MÜLLER-EBERHARD, KUNKEL AND FRANKLIN[7]. The latter designation more or less corresponds to the name "β_{2M}-" or "β_2-macroglobulin", which is used in the present paper.

β_{2B}-globulin is the name given by SCHEIDEGGER[8] to a weak precipitation line corresponding to a hitherto unidentified protein whose pathology is entirely unknown.

β_{2X} is the temporary name which we use for an equally unknown trace component visible in all sera.

γ_X-globulin is the name we use for a precipitation line in the β_2 and γ regions which is absent from normal sera. It can be observed in many inflammatory and neoplastic diseases. Its relationship to known proteins is still under study.

Relationships between γ, β_{2A}- and β_{2M}-globulins: The immuno-globulin concept

The three first proteins of the preceding enumeration are not without mutual resemblances. They will now be envisaged from chemical, physical, immunological and biological standpoints. (Table I)

Chemical properties: All three are completely precipitated by 2*M* ammonium sulfate. γ- and β_{2M}-globulins are of a pronounced euglobulin character, which is absent in β_{2A}.

All three contain some carbohydrate, as do most serum proteins. The carbohydrate content of γ-globulin is very low (about 3%) with some increase in fractions

References p. 646

of higher mobility[9]. On the other hand, the carbohydrate moiety of both β_{2A}[5] and β_{2M} seems to be of the order of 10%. It thus appears that the P.A.S. positive material found in the classical γ area would in part be accounted for, not by "immunologically" defined γ-, but by β_{2A}- and β_{2M}-globulins.

Physical properties: Electrophoretical investigations of isolated β_{2A}-globulin[5] showed it to move distinctly faster than the main γ peak. It thus appears to have the the same mobility on paper as the heavy β_{2M}.

TABLE I

A COMPARISON OF γ-, β_{2A}- AND β_{2M}-GLOBULINS

Protein	*γ-Globulin*	*β_{2A}-Globulin*	*β_{2M}-Globulin*
Synonyms	7 S γ; γ_2	β_2-macroglobulin; 19 S γ; γ_1	—
References	7	7	5*
Mobility (on paper)	from β_1 to γ	β_2	β_2
Sedimentation constant	7 S	19 S	7 S
% Hexose	1.22	5.20	4.75
% Fucose	0.29	0.62	0.22
% Hexosamine	1.14	2.90	3.75
% Sialic acid	0.22	1.70	1.74
% Carbohydrate	2.58	9.80	10.66
Ratio $\frac{\text{hexosamine}}{\text{hexose}}$	0.94	0.55	0.79
% Nitrogen	15.64	14.47	16.20

* Ultracentrifugal examination as well as fucose and sialic acid determinations of β_{2A}-globulin were performed through the courtesy of Prof. H. E. SCHULTZE (Behringwerke, Marburg, Germany).

Ultracentrifugal data[5] showed the identity of the sedimentation constants of γ- and β_{2A}-globulins which are both 7-S components, in contrast to β_{2M}-globulin which is a macromolecular 19-S component.

Immunological properties: Antigenic relationships between β_{2M}- ($=\gamma_1$-) and γ- ($=\gamma_2$)- globulins have been emphasized by FRANKLIN AND KUNKEL[10]. With certain antisera extensive cross-reaction between both proteins could be shown.

Similarly we mentioned[5] cross-reactions between isolated β_{2A}- and γ-globulins. It would seem that horse antisera against human serum show only very limited cross reactivity between both proteins, while for rabbit antisera the opposite was found in many samples tested. Thus it is relatively easy to absorb horse antiserum with isolated β_{2A} preparations without impairing reactivity against γ-globulin, while the same selective absorption is rather delicate for rabbit antisera. Possibly this may be due to the fact that horses possess some antigenic determinant which is shared both by human γ- and by human β_{2A}-globulin. They would thus be rather insensitive towards the common antigen, to the profit of more specifically β_{2A}- and γ-antigenic determinants. Horse serum when investigated immuno-electrophoretically* shows a very long γ-globulin line as well as a strong bow due to the so-called "T-component", whose shape, position and partial confluence with the γ-line are very strongly reminiscent of human β_{2A}-globulin.

* The author is indebted to Prof. H. E. SCHULTZE, Behringwerke, Marburg, for the kind gift of rabbit anti-horse antiserum, which allowed this investigation.

References p. 646

SCHEIDEGGER AND BUZZI[11] recently published immunological data on the antigen mosaic of human γ-globulin, and also confirmed that β_{2A} shared some common antigenic group with γ.

With many antisera we observed that the faint inner reduplication of the γ-bow, which we called "γ_B", was regularly interrupted at the point where this bow met the β_{2A}-line (Fig. I), and therefore we think that it is this faint inner bow which carries the common antigenic determinant.

A situation more or less comparable to the complexity of the human β_{2A}-γ

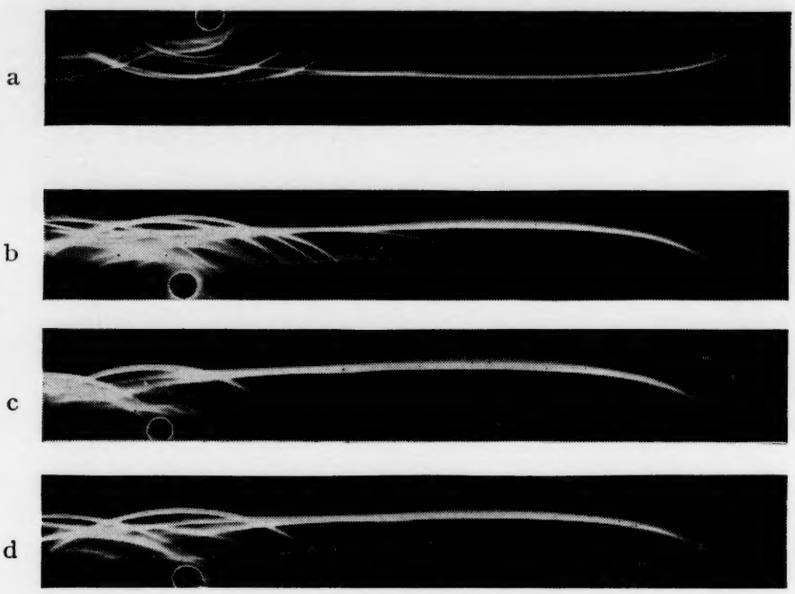

Fig. 2. Simultaneous increase of all immunoglobulins *a*. Normal serum. *b*. Serum from a case of severe chronic tuberculosis. *c*. Serum from a case of rheumatoid arthritis. *d*. Serum from a case of liver cirrhosis.

system seems to exist in mouse serum where three components, called- γ-, $\beta_{3\text{-II}}$ and $\beta_{3\text{-II}}$ may be seen very closely associated[12].

Biological properties: The association of antibody activity with γ-globulin is too well-known to be discussed here. In recent years many antibody activities, however, have been found to be carried by components of high molecular weight, which were generally demonstrated to have the properties of β_{2M}-globulin. This is true for cold agglutinins[13], saline-type Rh agglutinins[14], isoagglutinins[15] (to some extent at least), heterophilic antibodies, etc. As mentioned by KUNKEL[16], this heavy β_{2M}-globulin seems to have a more specialized function of carrying antibodies directed to red cells. The rheumatoid factor which has been widely studied recently, appears to be identified with, or to contain, β_{2M}-globulin[17].

The marked resemblances between the antibody-rich T-component of the horse and human β_{2A}-globulin made a search for antibody activity in the latter protein imperative. Through the courtesy of Prof. SCHULTZE (Marburg), samples of β_{2A}-globulin obtained by the zinc fractionation method[5] were tested and found to contain various antibodies. These studies are still in progress.

The immunoglobulin concept: All the data discussed above seem to point to the existence of a system of closely related, though not identical, proteins which are capable of acting as antibodies. These are: (a) *γ-globulin* (7S, low carbohydrate content,

References p. 646

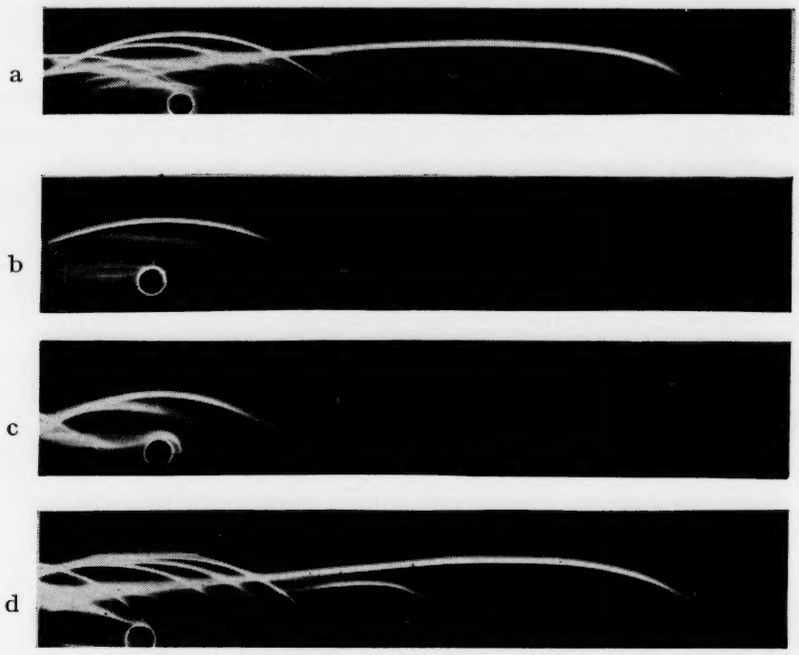

Fig. 3. Diseases associated with immune paralysis. *a*. Normal serum. *b*. Serum from a case of primary agammaglobulinemia in a child. *c*. Serum from a case of secondary hypogammaglobulinemia (HODGKIN's disease). *d*. Serum from a case of lymphatic leukemia.

heterogeneous mobility), itself split into two subfractions: γ_A, the major part, and γ_B, the minor component; (b) β_{2A}-*globulin* (also 7S, high carbohydrate content, high mobility), sharing many antigenic determinants with the γ, and especially with the γ_B component; and (c) β_{2M}-*globulin* (19 S, high carbohydrate content, high mobility).

The outlined similarities in nature and function clearly call for the adoption of a common name for all these substances. A word such as "immunoglobulins" would seem to be suitable. It will be seen in the next section that many observations on pathological conditions are in good agreement with such a view.

THE PATHOLOGY OF THE IMMUNOGLOBULINS

Several syndromes may be distinguished where the behaviour of the three immunoglobulins imprints distinct features on the clinical picture or on the laboratory data.

Simultaneous increases of the immunoglobulins

Three main conditions are classically associated with an important increase of the γ peak of paper electrophoresis strips, where that increase affects the γ area as a whole (so-called broad-γ-band syndromes). These are: (a) Chronic infections, especially tuberculosis, endocarditis lenta and osteomyelitis of long standing; (b) Collagen diseases, especially disseminated lupus and rheumatoid arthritis; (c) Liver diseases, especially cirrhosis.

Rarer diseases, such as chronic glomerulonephritis, WALDENSTRÖM's hyperglobulinemic purpura, etc. also belong to this class.

In almost every instance, immuno-electrophoresis shows a simultaneous increase of all three immunoglobulin fractions, irregularly associated with modifications

References p. 646

in the other serum proteins. The parallelism between γ, β_{2A} and β_{2M} is not always very strict, and it is our impression that particularly high increases of both β_{2A} and β_{2M} may be responsible for the so-called β-γ fusion which is often observed in cirrhotic sera. In addition, new splittings of the γ-line may be seen, especially in liver disease and hyperglobulinemic purpura.

The significance of this immunoglobulin reaction in infectious diseases seems rather obvious. In collagenoses it is strongly reminiscent of the frequently observed synthesis of abnormal tissue or blood cell antibodies. As to the immunoglobulin reaction in liver diseases, the author wishes to avoid speculation on its possible pathogenic significance, but study on this subject is highly indicated.

Diseases associated with immune paralysis

In primary "agammaglobulinemia" the relative absence of all three components of the immunoglobulin system is a striking fact which has several times been mentioned in the literature[18]. Indeed, the reduction of the true γ-globulin levels often seems to be less severe than for β_{2A} and β_{2M} which generally disappear altogether (Fig. 3b).

A very interesting case of apparently congenital immune paralysis was recently described by GIEDION AND SCHEIDEGGER[8], where γ-globulin levels were quantitatively normal, but where both β_{2A} and β_{2M} were lacking from the serum.

In acquired immune paralysis, which is generally secondary to some proliferative disease of the reticulo-endothelial system or its derivatives, various important and discordant disturbances in the immunoglobulin system are possible. Most often the γ-levels are very low and β_{2A} as well as β_{2M} are found to be absent (Fig. 3c). The author has seen a case of severe acquired immune paralysis of unknown origin where β_{2A} and β_{2M} were apparently normal, but where γ-globulin seemed to be entirely absent. Sometimes marked increases in one or two of the immunoglobulins associated with severe reduction of the other component(s) may be encountered (Fig. 3d).

All these facts suggest that various protein-synthesizing mechanisms normally operate to provide the organism with a sufficient spectrum of antibodies, and that in pathological conditions either one or several of these antibody production lines may be interrupted. For this reason the term of "agammaglobulinemia" seems to be a rather unfortunate choice of words.

Paraproteinemias

Each of the three main immunoglobulin fractions: γ, β_{2A} or β_{2M} may give rise to so-called paraproteins. In such cases the fraction involved is subjected to an extremely high increase associated with various qualitative abnormalities. An additional feature of those diseases producing paraproteins is the very frequent and often severe depression of the synthesis of the two immunoglobulins not involved in the paraprotein transformation. The same applies to uninvolved electrophoretic portions of the affected immunoglobulin. These findings are probably at the basis of the frequently observed immune paralysis in such patients.

For *multiple myeloma*, the γ-globulin nature of most paraproteins has been suspected since a long time. It was definitively demonstrated by GRABAR's immunoelectrophoresis[19]. The latter technique also showed that in addition to the well-known paraprotein peak minor abnormalities of the most diverse nature could occur in the

References p. 646

γ-globulin. Also the β_{2A}-globulin showed abnormal features ranging from near-disappearance to marked increase.

A group of myelomas where the paraprotein obviously cross-reacted with the normal γ-antigen only slightly or even not at all was recognized by various writers[20,21]. It was suspected[21] that such paraproteins derived from unidentified normal β-globulins. We were recently able to make a thorough study of such cases by using antisera specifically absorbed with β_{2A}-globulin, and by the use of specific anti-β_{2A} antiserum. It appeared that there existed a group of myelomas where the paraprotein peak was

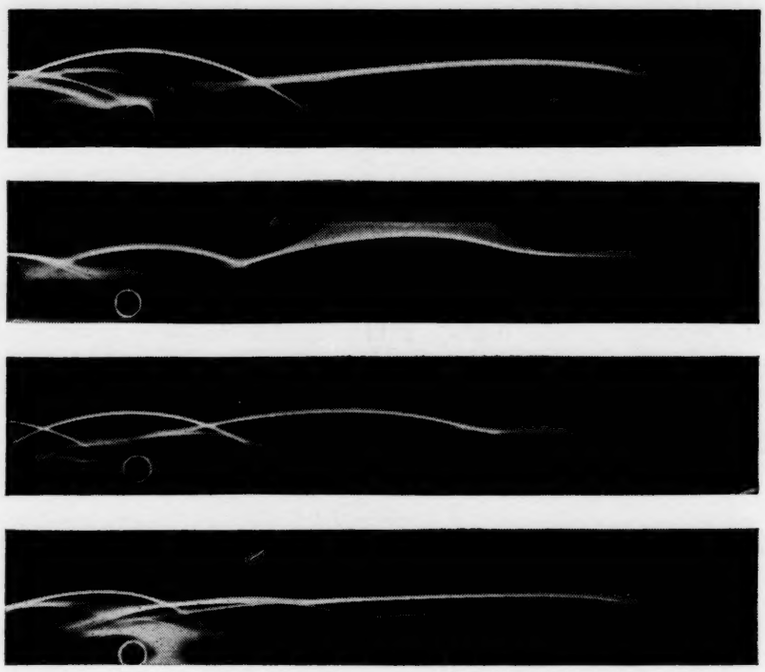

Fig. 4. The three main types of paraproteinemia. Each of the three immuno-globulins may give rise to a paraprotein. Paraproteins derived from γ-globulin or from β_{2A}-globulin are characteristic for multiple myeloma. Macroglobulinemia WALDENSTRÖM derives its paraprotein from the β_{2M}-globulin. *a*. Normal serum. *b*. Serum from a case of γ-myeloma (with β mobility on paper). *c*. Serum from a case of β_{2A}-myeloma (also β on paper). *d*. Serum from a case of macroglobulinemia.

wholly constituted by β_{2A}-globulin. In such cases the γ-levels were often strikingly low. The name of "β_{2A}-myelomas"[22], instead of "γ-myelomas", seems to be suitable to describe such cases. Additional forms of myeloma may be encountered where the paraprotein seems to derive entirely from the antigenic groups which are common to both γ- and β_{2A}-globulins, without possessing either of the specific γ- or β_{2A}-antigens. The paraprotein precipitation line can then be inhibited equally well by absorption of the antiserum with γ- or with β_{2A}-globulin.

It should be stressed that no clinical or morphological differences seem to exist between those various myeloma forms. On the other hand they present striking chemical discrepancies, such as hypernormal or low-normal zinc sulfate turbidity tests and high paraprotein carbohydrate for the β_{2A}-myelomas, as opposed to high zinc turbidity values with low paraprotein carbohydrate for those derived from the γ-antigen.

Macroglobulinemia WALDENSTRÖM finally was found to derive its paraprotein from the physiologic β_{2M}-globulin[4]. Complicated associations of β_{2M}- and γ-globulins

References p. 646

in chemical bond were found by the author often to occur in macroglobulinemia sera. Their possible biological significance is still under study.

SUMMARY

Three serum proteins have been selected for this study because of their close chemical, immunological and functional relationships. These are γ-globulin, β_{2A}-globulin and β_2-macroglobulin.

They appear to be the carriers of antibody activity and we propose to call them by the name of *immunoglobulins*.

In chronic infections, collagen diseases and cirrhosis (as well as hepatitis) all three immunoglobulins increase markedly in serum. This may be correlated to antibody synthesis.

Diseases associated with immune paralysis (primary and secondary agammaglobulinemia and biologically related syndromes) are characterized by the fall of at least one of the three immunoglobulins.

Diseases with uncontrolled proliferation of cell types derived from the reticulo-endothelial system (myeloma, macroglobulinemia, reticulo-sarcoma, leukemias) may give rise to "paraproteins", which invariably appear to be derivatives of one (sometimes two) of the immunoglobulins. There seems to be a rule that in such cases the production of those of the immunoglobulins which take no part in the paraproteinemia is markedly reduced.

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A PERIODIC ACID SCHIFF-STAINING MACROMOLECULAR COMPONENT OF HUMAN HEPATIC BILE*

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INTRODUCTION

In the course of investigations carried on in this laboratory on a mucoprotein component of human urine[1-3] with a view to elucidating some of the mechanisms possibly involved in the formation of urinary calculi, it seemed appropriate to examine human bile to see if a component with similar solubility properties could be isolated, since similar mechanisms may be involved in the formation of biliary calculi. Accordingly, several samples of hepatic bile were obtained from a patient following cholecystectomy. A macromolecular component has been isolated and studied with paper electrophoresis, ultracentrifugation, absorption spectroscopy and electron microscopy.

MATERIALS AND METHODS

Hepatic bile was obtained as T-tube drainage from an 82-year-old woman who had had a cholecystectomy and common duct exploration for gall stones following repeated episodes of biliary colic and evidence of cholecystitis. The T-tube was inserted in the common duct. Several daily samples of bile were so obtained.

Ultracentrifuge studies were done in a Spinco analytical ultracentrifuge at 20° in a single-sector cell. Paper electrophoresis runs were made in a Kunkel zone-paper electrophoresis apparatus[4]. Absorption spectra were measured in a Beckmann recording spectrophotometer. Viscosities were measured with a modified Ostwald capillary viscometer. Refractive indices were measured in a Brice-Phoenix differential refractometer.

For paper electrophoretic examination, hepatic bile was dialyzed with frequent changes against distilled water for several days, concentrated to about $^1/_5$ of its volume by suspending the dialysis bag before a fan, and finally dialyzed against barbital buffer pH 8.6, ionic strength (μ) 0.1. After centrifuging to clarify the solution, paper electrophoresis strips were prepared and stained individually with PAS (periodic acid Schiff stain)[5], bromphenol blue[4], and azocarmine[6], and examined for fluorescence. For a detailed description of paper electrophoresis strips prepared from bile in this way, see the work of VERSCHURE[6].

RESULTS

The paper electrophoretic boundary which remains at the starting line was found to stain positively with PAS. Bromphenol blue and azocarmine stain the strips weakly in this region but such strips fluoresce red and orange, respectively, near the starting

* Research supported by the U. S. Atomic Energy Commission.

References p. 651

line. The material comprising this boundary must have been either uncharged or in the form of large aggregates which could not migrate in the paper.

Accordingly, to isolate the aggregates the barbital solution prepared as above was centrifuged at 40,000 rev./min in a Spinco preparative ultracentrifuge for 1–$^1/_2$ h. A clear bluish gel was obtained overlaying a yellow precipitate. The gel dissolved more readily in distilled water than the yellow precipitate which was then removed by centrifugation at low speed. Paper electrophoresis of this final supernate dialyzed against barbital buffer pH 8.6, μ 0.1, showed a single stationary boundary which was intensely PAS positive.

The gel from barbital solutions was found to be readily soluble in water but insoluble in 0.1 *M*, 0.6 *M*, and 1.0 *M* NaCl. Ultracentrifuge studies were therefore performed in water and showed (Fig. 1) a single component represented by an

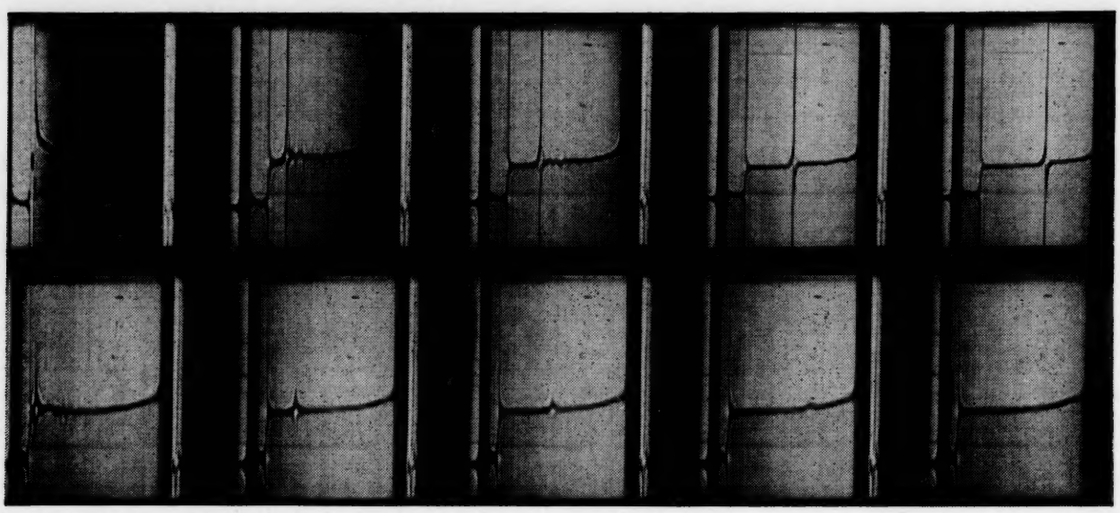

Fig. 1. Ultracentrifuge patterns of biliary mucoprotein. Photos at 16-min intervals; bar angle top from left to right 45°, 45°, 50°, 55°, 55°, bottom all photos at 40°; speed 52,640 rev./min; temperature 20°; concentration top 1.62 mg/ml, bottom 0.70 mg/ml.

extremely sharp peak. The peak did not split even at low concentration where the molecular interaction would have a lesser tendency to hold two boundaries together. Some polydisperse, rapidly sedimenting material is visible in the first photograph taken within a few seconds of the time when the centrifuge reached full speed. After this material was removed by centrifugation and the supernate examined in the ultracentrifuge, the heavy material reappeared, suggesting that it may be produced by aggregation of the lighter component. A dilution series of ultracentrifuge runs were made to obtain a linearly extrapolated sedimentation constant in water of 22.6 S. The extrapolation of ultracentrifuge data obtained in distilled water is subject to much uncertainty. The primary data are shown in Table I where the marked concentration dependence of the sedimentation velocity is apparent. The seven points of lowest concentration were extrapolated linearly to obtain the sedimentation constant. A solution of concentration 0.73 mg/ml had a sedimentation velocity of 12.4 S and a relative viscosity of 1.35 which corrects the sedimentation velocity to 16.8 S; still far below the extrapolated value. The samples used in these runs had been stored at 1° and extensively dialyzed for nearly two months. Two ultracentrifuge runs on preparations which had not been so extensively dialyzed against water showed more of the

References p. 651

rapidly sedimenting material and single sharp peaks of similar appearance to those of the dilution series, but with sedimentation velocities of 21.3 and 18.6 S. Although these runs were made at a temperature somewhat higher than 20, the sedimentation velocities are greater than would be expected. These variations in sedimentation velocity are due to variations in the electroviscous effect with slightly different salt

TABLE I

MUCOPROTEIN OF BILE
ULTRACENTRIFUGE DATA

Concentration mg/ml	*Sedimentation velocity · 10^{+13} cm/sec/dyne*
1.59	7.68
1.52	8.02
1.45	8.18
1.38	8.68
0.80	11.20
0.78	11.81
0.74	12.26
0.69	12.82
0.68	12.89
0.64	13.62
0.59	14.34

Extrapolated sedimentation constant
$= 22.6 \cdot 10^{-13}$ at zero concentration.

concentrations at very low ionic strength, rather than to different states of aggregation. The evidence for this is that the extrapolated values of the sedimentation constants are 23 S or slightly higher, whereas the measured sedimentation velocities show a wide variation. A single attempt to obtain ultracentrifuge data in 0.1 *M* NaCl solution before aggregation by adding the salt just before loading the cell showed no sharp peak characteristic of this material. Only the diffuse, rapidly sedimenting peak was visible. This is further evidence that the diffuse peak represents aggregates of molecules of the sharp component and that the aggregation is reversible. These aggregates are not completely dispersed into individual molecules even in distilled water. In 0.1 *M* salt the molecules are quickly and completely aggregated. From the diffuseness of the aggregate peak, one would conclude that the aggregates are not all of the same size but contain varying numbers of molecules.

Ultraviolet absorption spectra obtained from the barbital precipitate dissolved in water show an absorption maximum at 276 mμ characteristic of aromatic amino acid containing proteins. The spectra are otherwise remarkable only in showing a great deal of scattering presumably from the above-mentioned aggregates. Much of the pigment in bile which cannot be removed by dialysis against water can be removed by dialysis against barbital. The pigment is therefore bound to macromolecules and can be displaced by barbital. The barbital in turn may then be bound and contribute its absorption to the observed spectrum. Indeed, the observed spectrum of the barbital precipitate had a shoulder at 260 mμ even though it was measured against dialysate as a blank. Accordingly, a sample of bile dialyzed against water was

References p. 651

prepared by three times repeated precipitation with 0.1 *M* NaCl and resuspension in water. This sample was never exposed to barbital. The solution remained yellow in contrast to similar solutions prepared with barbital which were water-clear. The spectrum showed an absorption maximum at 276 mμ but none at 260 mμ, indicating that the 260 mμ absorption was due to bound barbital rather than to a naturally occurring purine or pyridine-pyrimidine part of the macromolecule. Again the absorption rose rapidly at shorter wavelengths, suggestive of intense scattering. The ultracentrifuge pattern of material prepared with NaCl is similar to that prepared with barbital except for a greater proportion of aggregated material due to the lower pH and the shorter period of dialysis against water.

The large sedimentation constant obtained above indicates a very large molecular weight even when allowance is made for the high density of mucoproteins. This is confirmed by electron microscope observations which show a multitude of nearly spherical particles measuring 150 Å in diameter. In the electron micrographs some of the particles are dispersed singly but most are aggregated in clumps about 600 Å to 1000 Å in diameter. The clumps vary considerably in size and in the number of molecules which they contain. The individual particles and the clumps seen in the electron micrographs can be associated with the sharp peak and the diffuse rapidly sedimenting peak, respectively, seen in the ultracentrifuge.

DISCUSSION

The isolation of this macromolecular component from several different samples of bile from the patient studied is highly reproducible. The degree of purity and homogeneity of the material is sufficient for most physicochemical and biochemical methods of study, although the aggregates would interfere seriously with light scattering. The sharpness of the ultracentrifuge peak is attributable to the molecular interaction to be expected in water solution with an expanded ion cloud about each macromolecule and does not indicate the presence of fibrous molecules. Similarly the high relative viscosity and the great dependence of sedimentation velocity upon concentration are understandable in terms of the electro-viscous effect rather than high assymetry of the molecule.

An ever-present question is whether a component isolated from a biological substance has been modified by the isolation procedure. In the present case the isolation procedure was extremely gentle; the pH did not go above 8.6 or below 5.5; the ionic strength was always below 0.1 and no reagents other than barbital, NaCl, and water were added. This argues for the belief that the mucoprotein is indeed in its native state.*

The mucoprotein nature of the macromolecules may be deduced from their ultraviolet absorption spectrum, their property of staining positively with PAS, their very large size, their solubility properties and their faint staining with bromphenol blue and azocarmine B.

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* The state of freshly secreted unfractionated bile is, however, somewhat different from that of bile which has been stored. This was demonstrated by measuring the viscosity of fresh bile at various intervals following a one-half hour period of collection. The relative viscosity was 1.18 when first measured 1–½ h after the end of the collecting period. It dropped rapidly to 1.15 in another ½ h, and then dropped slowly to 1.12 during a period of 2 days. All measurements were made at 20°.

References p. 651

This component in bile resembles the urinary mucoprotein of TAMM AND HORSFALL in its staining properties, its solubility characteristics in water and in dilute salt solution, and its tendency to form aggregates in dilute salt solution, but is very different in shape.

The mucoprotein studied here can be identified with protein component P_4 of VERSCHURE[6] by its behavior on paper electrophoresis. VERSCHURE reported that it was present in 4 patients from which fistula bile was obtained, in all samples of gall bladder bile from cholecystitis patients, in no bile from normal gall bladders, and almost constantly in bile samples from autopsies. He studied fistula bile from 27 patients and 31 samples of gall bladder bile. The present patient had cholecystitis and gall stones. Thus the presence of the mucoprotein is presumably correlated with the occurrence of cholecystitis and gall stones.

SUMMARY

A method has been developed for isolating and purifying a macromolecular water-soluble component from human hepatic bile by precipitation from barbital solution. The purity and homogeneity of the component have been demonstrated by paper electrophoresis, ultracentrifugation and electron microscopy. The component is a mucoprotein composed of nearly spherical macromolecules 150 Å in diameter with an extrapolated sedimentation constant in water of 22.6 S. The presence of the mucoprotein in human bile may be correlated with the occurrence of cholecystitis and biliary calculi.

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THE DETERMINATION OF BILE ACIDS IN BILE AND DUODENAL CONTENTS BY QUANTITATIVE PAPER CHROMATOGRAPHY

BILE ACIDS AND STEROIDS 71

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Four years ago a method was published for the quantitative determination of mixtures of bile acids separated by paper chromatography[1]. With suitable solvent systems this method allowed the determination of different free bile acids as well as their corresponding taurine and glycine conjugates. The method was therefore thought to be of value for the differential analysis of the bile acids occurring naturally in bile and intestinal contents. Preliminary reports have been published of the modifications necessary for this purpose[2, 3]. The method described in this paper has now been used for three years at this Institute in different studies of the bile acid metabolism of man, rats and rabbits.

EXPERIMENTAL

The original method has been subjected to several modifications, mostly because of the difficulties encountered in its application to biological material.

Solvents

Glass-distilled water. Ethanol, 99.5% spectrographically pure, and 96% redistilled. Acetic acid, p.a. 97–99% (Merck), redistilled. Formic acid, p.a. 98–100% (Merck), redistilled. Ethylene chloride, puriss. (Fluka). Shaken with concentrated sulfuric acid, washed with water, dried over K_2CO_3 and distilled. Isoamyl acetate, puriss. (Riedel de Haen) redistilled. *n*-Heptane, puriss. (May and Baker) redistilled.

Preparation of paper

Whatman 3 MM filter paper was used throughout. The sheets were cut as previously described[1]. By removing three rectangular pieces of paper 10 × 355 mm from a paper 90 × 465 mm, four separate strips 15 × 355 mm were obtained, held together by intact paper at both ends. At one end this intact paper was 90 × 80 mm (upper end) and at the other, 90 × 30 mm (lower end). For ascending chromatography, a starting line was drawn 30 mm from the lower end of the separate strips, and for descending chromatography this line was drawn 25 mm from their upper end. Since in the latter case the moving phase was to drip off the lower edge of the paper, this edge was symmetrically cut as a saw; the teeth were 5 mm broad at their base and 10 mm long.

Six papers were hung in an ordinary tank for descending chromatography (3 on each side of the trough). They were washed with different solvents in the following order: 400 ml of 96% ethanol; 400 ml of 0.5 *N* hydrochloric acid; 400 ml of glass-

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References p. 664

distilled water; 400 ml of 96% ethanol; 150 ml of isoamyl acetate; and 400 ml of 96% ethanol. The solvents were continuously supplied to the trough from a tightly stoppered separating funnel. The level of solvent in the trough was adjusted to give a relatively slow flow of solvent through the papers and the whole procedure was completed in 3–4 days. Each solvent was allowed to drain completely before the next was added.

Immediately before use, the papers were taken out and dried at 100°. When they were not used immediately, they were hung in the tank and washed with 150 ml of 96% ethanol every day.

Paper chromatography

For details see SJÖVALL[4, 5]. A suitable amount of bile or duodenal contents was put on the starting line of three of the paper strips. The fourth strip was used as a blank. The choice of chromatographic solvents depended upon the nature of the bile acids present in the material to be analyzed. The phase systems used and their applicability to various bile acid mixtures are summarized below. All chromatography was carried out at 23° ± 1°. Equal parts of the moving and stationary phases were equilibrated before use by shaking and separating at this temperature.

Phase system G_a. Moving phase: 70% (v/v) ethylene chloride–heptane; stationary phase: 70% (v/v) acetic acid–water. Papers were equilibrated for 8–16 h in the tank with the vapors of both phases before being brought into contact with the moving phase. Ascending chromatography for 6–8 h. Used for the separation of glycine-conjugated bile acids when glycochenodeoxycholic and glycodeoxycholic acids were not present simultaneously.

Phase system G_d. Moving phase: 50% (v/v) ethylene chloride–heptane; stationary phase: as G_a. Equilibration in the tank as with system G_a. Descending chromatography for 18 h. Used for the separation of glycine-conjugated bile acids when both glycochenodeoxycholic acid and glycodeoxycholic acid were present.

Phase system T_a. Moving phase: 85% (v/v) isoamyl acetate–heptane; stationary phase: 70% (v/v) formic acid–water. Papers equilibrated for ½ h in the tank with the vapors of the moving phase before being brought into contact with this phase. Ascending chromatography for 20 h. Used for the separation of taurocholic acid from the taurine-conjugated dihydroxycholanic acids when no separation of the latter acids was needed.

Phase system T_d. Moving phase: 80% (v/v) isoamyl acetate–heptane; stationary phase: as G_a. Papers equilibrated in the tank as with system T_a. Descending chromatography for 40 h. Used for the separation of taurine-conjugated dihydroxycholanic acids (see also Results and Discussion).

Phase system F_a. Moving phase: 40% (v/v) ethylene chloride–heptane; stationary phase: as G_a. Papers equilibrated for about 16 h in the tank with the vapors of both phases before being brought into contact with the moving phase. Ascending chromatography for 6 h. Used for the separation of free bile acids when chenodeoxycholic and deoxycholic acids were not present together.

Phase system F_d. Moving phase: 20% (v/v) ethylene chloride–heptane; stationary

References p. 664

phase: as G_a. Papers equilibrated in the tank as with system F_a. Descending chromatography for 18 h. Used for the separation of free bile acids in the presence of both chenodeoxycholic and deoxycholic acids.

Elution of bile acids from the chromatograms

When the chromatograms had been run, they were dried at room temperature for ½–1 h. The bile acids on one of the strips of the ascending chromatograms were revealed by spraying with a 15% ethanolic solution of phosphomolybdic acid and heating for a few minutes at 80°. With these spots as a guide, the bile acid zones on the other strips were cut out, as well as corresponding zones on the blank strip.

In the descending chromatograms, however, the bile acids did not move the same distance on all three strips. Therefore two strips were sprayed, leaving an unsprayed strip between them. It was found that the rate of travel of the bile acids on the latter strip was the mean of the travelling rates of the corresponding bile acids on the two sprayed strips. Thus the bile acid zones on the unsprayed strip could be accurately cut out. Corresponding paper pieces were also cut out from the blank strip.

The paper pieces containing the bile acids were eluted with spectrographically pure ethanol in the apparatus previously described[1]. Care had to be taken that the part of the paper piece containing the bile acid was not clamped between the microscopic slides during the elution, as this would result in incomplete elution of this part. After elution the eluates were evaporated overnight at 100°.

Determination of the bile acids eluted

65% sulphuric acid prepared as previously described[1] was used throughout. The samples were dissolved in 1 ml of this acid by shaking thoroughly for 5 min in a shaking apparatus. If hot, the samples were cooled to room temperature under tap water before the measurements. The measurements were carried out against the corresponding blank sample in a Beckman DU spectrophotometer. Absorption spectra were recorded with a self-recording spectrophotometer (Perkin-Elmer, Spectracord 4000). Parallel measurements of known amounts of synthetic bile acids or their sodium salts were always carried out. A summary of the conditions used for the determination of the different bile acids is given in Table I.

TABLE I

*Bile acids**	*Min in sulphuric acid*	*Temperature °C*	*Measured at wavelengths (mμ)***
TC	60	20	320; 389
TCD	15	60	305; 389
TD	10	60	308; 389
TCD + TD	15	50	305; 389
GC	60	20	320; 389
GCD	15	60	305; 389
GD	10	60	308; 389
C	60	20	320; 389
CD	60	60	380; 310
D	60	60	385; 310

* C = cholic, CD = chenodeoxycholic, D = deoxycholic acid, prefix T = tauro-, G = glyco-.

** The extinction at the wavelength first mentioned was used for the calculation of the amount of acid present.

References p. 664

The contribution of substances other than bile acids to the light absorption in sulphuric acid was corrected for by measuring the extinction of a corresponding sample in 80% ethanol (against 80% ethanol). The extinctions obtained were subtracted from the extinctions in sulphuric acid at the corresponding wavelengths. The resulting values were used for the calculation of the amount of bile acid present. This correction always had to be carried out for the taurine-conjugated acids and for glycine conjugates separated with phase system G_a. When the bile acid concentration was low, a similar correction had to be made for glycocholic acid eluted from chromatograms with phase system G_d. The very small amounts of free bile acids sometimes present under pathological conditions also had to be corrected, whereas in the case of free bile acids obtained after hydrolysis no correction was necessary.

RESULTS AND DISCUSSION

Paper chromatography of bile and duodenal contents

The direct use of unpurified bile and duodenal contents for paper chromatography greatly shortens the time needed for analysis. Except with phase system F_d, well-defined spots of the bile acids are obtained even when the single bile acids are present in amounts less than 1% of the total solids. A bile acid can be detected in a concentration of about 1 mg/100 ml bile, but in this concentration range quantitative measurements are only approximate.

The use of washed filter papers, which is necessary for the quantitative measurements, somewhat altered the chromatographic behaviour of bile acids in biological materials. This was particularly the case with the taurine-conjugated acids. With the phase systems earlier described, these acids did not separate as well as on the unwashed papers, especially when large amounts of interfering substances were present. Phase system T_a was therefore worked out and by its use good separations of taurocholic acid from the taurine-conjugated dihydroxycholanic acids were always obtained. A separation of taurochenodeoxycholic acid from taurodeoxycholic acid on washed papers with phase system T_d could, however, be achieved only when the concentration of bile acids in the material to be analyzed was high, *e.g.* in normal gall-bladder bile or in duodenal contents after the intravenous administration of cholecystokinin. Separation on unwashed papers according to SJÖVALL[5], could be carried out even when the bile acid concentration was relatively low, but no quantitative measurements could be made in this case.

The separation of glycine-conjugated bile acids in biological materials was not appreciably affected by washing the papers. The solvent flow in descending chromatography with phase system G_d was markedly faster than observed earlier with unwashed filter papers[5]. This was due not only to the previous washing of the papers, but also to the fact that they had been cut in narrow strips. The bile acid spots would streak if the solvent flow was too rapid and this was prevented by adjusting the amount of moving phase in the trough to a suitable level. The same phenomena were observed in the separations with phase system F_d.

Quantitative determinations

When the reference strips had been sprayed to reveal the positions of the bile acids, spots were also found that were not due to bile acids. This was usually the case only with the chromatograms of taurine conjugates, where weakly coloured spots

References p. 664

could also be seen on the unsprayed strips. Therefore whole strips from different chromatograms of bile and duodenal contents were cut in small pieces which were eluted separately. The absorption spectra of the eluates were recorded in 80% ethanol and in 65% sulphuric acid after heating at 60°. It was found that the spectra of the eluates of the different pieces were relatively similar in sulphuric acid and in ethanol except, of course, for the eluates containing bile acids. The spectra of some zones corresponding to spots of non-bile acid origin in a chromatogram with phase system T_a are shown in Fig. 1.

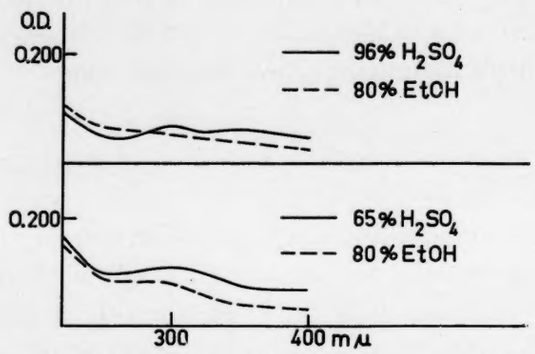

Fig. 1. Similarity between absorption spectra in 80% ethanol and in sulphuric acid of non-bile acid material from chromatograms with phase system T_a.

Since the absorption spectra in sulphuric acid and in ethanol are similar for the interfering substances appearing at the places in the chromatogram where no bile acids are present, it is reasonable to assume that they will be so at the places of bile acids too. Bile acids have no ultraviolet absorption in ethanol at the wave-lengths used. With phase system T_a at least two-thirds of the contribution of interfering substances to the extinction of the bile acids in 65% sulphuric acid was corrected for by subtracting the extinction in 80% ethanol. Similar corrections were usually made for the values obtained for bile acids separated with phase system G_a, and sometimes had to be made for glycocholic acid obtained from chromatograms with phase system G_d.

With this method of correction for interfering material, recovery experiments were carried out with synthetic bile acid mixtures before and after the addition of bilirubin glucuronide (kindly supplied by Dr. NOSSLIN) and an ethanolic extract of "white bile" (bile containing no bile acids or bile pigments, obtained from patients with cholecystitis). The results are shown in Table II.

TABLE II

QUANTITATIVE PAPER CHROMATOGRAPHY OF BILE ACID MIXTURES

with addition of bilirubin glucuronide (about 80 mg/100 ml) and an ethanolic extract of "white bile" (500 mg/100 ml). Each value is the mean of two determinations. Values for taurine-conjugated bile acids are corrected for "interfering substances".

Sample	*Bile acids, mg/100 ml*							
	*TC**		*TCD**		*GC**		*GCD**	
	calc.	*found*	*calc.*	*found*	*calc.*	*found*	*calc.*	*found*
Synthetic bile acids	81	80	159	162	186	203	343	340
Synthetic bile acids + bile pigment	81	89	159	179	186	180	343	359
Synthetic bile acids + "white bile"	93	103	154	154	–	–	–	–

* Abbreviations: See Table I.

References p. 664

Attempts to find a rapid method for the elimination of the interfering material from bile as well as attempts to change its chromatographic properties were made but were unsuccessful. The use of 96% instead of 65% sulphuric acid often produced erroneous results.

Determination of bile acids in rat bile

Cholic and chenodeoxycholic acids, conjugated mainly with taurine, are the main bile acids in rat bile (for references see BERGSTRÖM AND SJÖVALL[6] and NORMAN[7]). They are separated with phase system T_a. Table III shows the reproducibility of the quantitative analysis. Added bile acids can be quantitatively recovered.

TABLE III

REPRODUCIBILITY OF DETERMINATIONS OF THE DAILY BILIARY EXCRETION OF TAUROCHOLIC AND TAUROCHENODEOXYCHOLIC ACIDS IN BILE FISTULA RATS

Bile sample	*Number of determ.*	*Mg bile acid per day*			
		*NaTC**		*NaTCD**	
		Mean	*Range*	*Mean*	*Range*
GR 13 : 72	3	60.5	60.1–61.2	20.8	19.7–21.6
144	5	54.1	53.0–57.4	23.0	21.1–25.4
216	4	38.0	36.2–41.0	30.8	27.8–36.2
240	3	36.9	35.4–38.2	29.3	27.8–31.2
GR 16 : 72	2	28.9	28.9–28.9	11.4	11.3–11.5
96	2	28.2	27.6–28.7	15.3	14.8–15.7
120	2	31.5	31.5–31.5	13.1	12.5–13.7
144	2	44.5	44.4–44.5	22.9	21.8–23.9
168	2	32.2	31.8–32.5	10.3	10.1–10.5
192	2	26.6	26.4–26.7	19.6	19.5–19.7
216	2	14.7	14.6–14.8	15.0	14.9–15.0
240	2	21.0	20.8–21.2	34.1	33.6–34.5
GR 01/6	2**	50.5	49.7–51.6	20.5	19.9–21.0
GR A	3	42.2	38.8–44.2	39.5	38.5–40.7

* Abbreviations: See Table I.
** One year between the determinations.

Recently, two new bile acids have been isolated in rat bile[8]. A series of papers have been published by DOISY and his co-workers on the structure of these acids, Acid I and Acid II. They have been found to be 3α,6β,7β-trihydroxycholanic and 3α,6β,7α-trihydroxycholanic acids respectively[9]. Samples of these acids were kindly supplied by Professor DOISY and their sulphuric acid-absorption spectra were compared with that of cholic acid; the results are shown in Fig. 2, which also shows the spectrum of a similar acid, hyocholic acid, kindly supplied by Professor HASLEWOOD. This acid was observed in a paper chromatographic study of the bile acids of the hog[10], and was subsequently isolated by HASLEWOOD[11] and shown to be 3α,6α, 7α-trihydroxycholanic acid[12, 13].

As seen in Fig. 2, these acids have practically no light absorption under the conditions used for the quantitative determination of cholic acid. It is reasonable to assume that this will also be the case for the taurine conjugates of these acids which can be expected to travel with the same speed as taurocholic acid on the chromato-

References p. 664

grams. Thus, the quantitative determination of taurocholic acid in rat bile will not be influenced by the presence of Acid I and II. Further support for this view has been given by BERGSTRÖM AND DANIELSSON[14], who analyzed rat bile rich in Acids I and II by paper chromatography and by reversed phase partition chromatography.

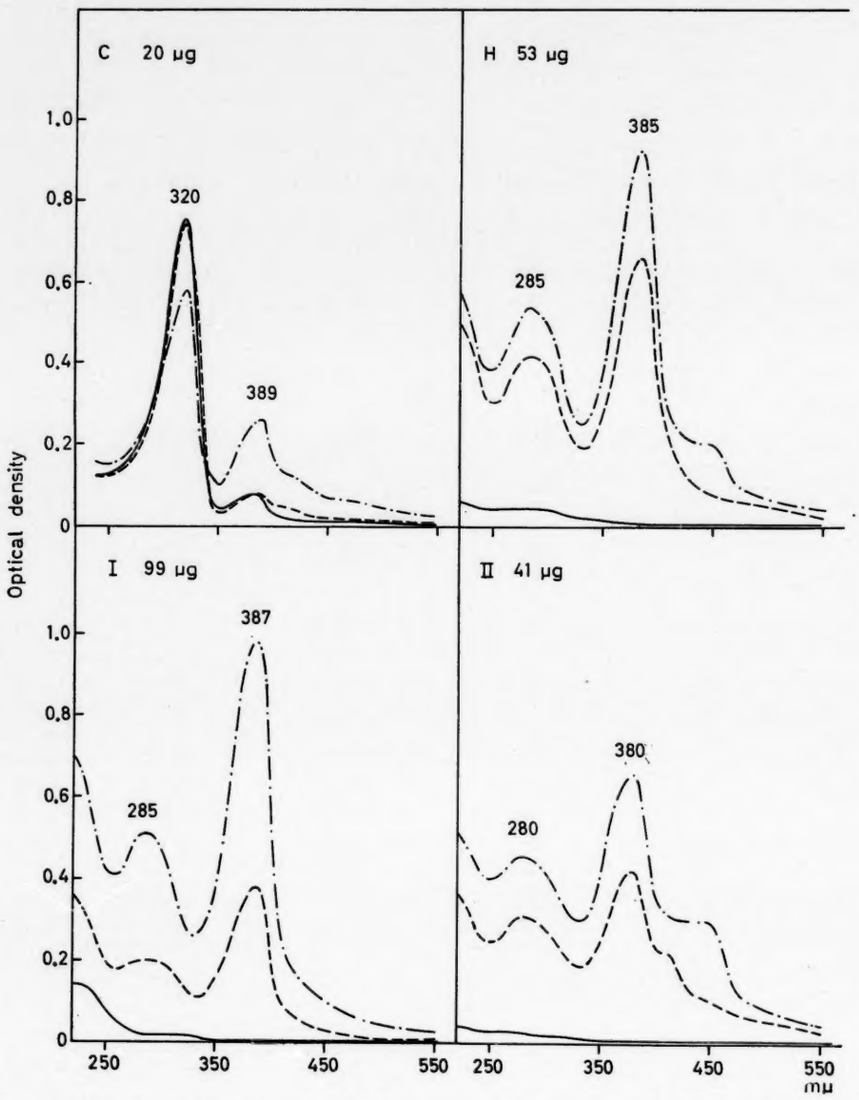

Fig. 2. Sulphuric acid absorption spectra of different trihydroxycholanic acids. Samples were dissolved in 1 ml of 65% sulphuric acid. (————): 60 min at room temperature; (– – –): 15 min at 60°; (–·–·–): 60 min at 60°. Hydroxyl groups in positions: 3α, 7α, 12α (C), 3α, 6α, 7α (H), 3α, 6β, 7β (I) and 3α, 6β, 7α (II).

Determination of bile acids in rabbit bile

Rabbit bile contains mainly glycodeoxycholic and glycocholic acids. These are separated with phase system G_a. Examples of the quantitative determinations are given in Table IV. Added bile acids are quantitatively recovered. For further details of the analysis of bile acids in rabbit bile see LINDSTEDT AND SJÖVALLL[15].

Determination of bile acids in bile and duodenal contents of man

The common bile acids occurring in man are cholic, chenodeoxycholic and deoxycholic acids (*cf.* WOOTTON AND WIGGINS[16]). They are conjugated with glycine and

References p. 664

TABLE IV

DETERMINATION OF BILE ACIDS IN A POOLED SAMPLE OF RABBIT FISTULA BILE EXCRETED DURING THE FIRST DAY AFTER THE OPERATION

Values are given for the amount of bile acids on the chromatograms and the bile acid concentration calculated. The values represent single determinations.

Amount of bile (μl)	*GC**		*GD**	
	μg	*mg%*	*μg*	*mg%*
10	1.8	18	32.6	326
	1.6	16	30.2	302
20	3.0	15	60.6	303
	3.0	15	65.6	328
40	6.6	17	131.5	329
	6.1	15	123.5	309

* Abbreviations: see Table I.

taurine, the former conjugates predominating. The simultaneous presence of deoxycholic and chenodeoxycholic acids necessitated working out methods for the determination of the conjugates of these acids after their separation with descending paper chromatography. Results for the determinations of mixtures of synthetic glycine conjugates separated with phase system G_d are shown in Table V. Except when present

TABLE V

QUANTITATIVE PAPER CHROMATOGRAPHY OF MIXTURES OF SYNTHETIC GLYCINE-CONJUGATED BILE ACIDS

Phase system G_d

Mixture of acids*	*Number of determinations*	*Amount (μg)*	*Per cent recovered*	
			Mean	*Range*
GCD + GD	4	21.2	97.2	93.6– 98.0
		21.7	98.8	98.0–100.6
GC + GCD + GD	4	22.3	96.6	93.5– 99.4
		20.4	103.6	100.0–109.4
		21.0	97.5	96.7– 98.2
GC + GCD + GD	4	22.3	95.8	94.5– 97.0
		10.2	94.5	88.7–100.4
		10.5	96.2	91.0–102.2
GC + GCD + GD	4	11.2	97.2	93.5– 99.4
		20.4	101.4	88.5–113.8
		10.5	98.2	94.9–104.0

* Abbreviations: see Table I.

in high concentrations, the taurine conjugates could not be analogously determined with phase system T_d; the reasons have been discussed in the section on paper chromatography. The decision was made, therefore, to determine the taurocholic acid and

References p. 664

the sum of taurochenodeoxycholic and taurodeoxycholic acids as they were separated with phase system T_a. Suitable conditions were worked out to get an identical molar extinction of the two taurine-conjugated dihydroxycholanic acids in sulphuric acid. The results are shown in Tables VI and VII.

TABLE VI

OPTICAL DENSITIES OF 26 μg NaTCD* AND NaTD* at 305 mμ in 1 ml 65% sulphuric acid after different heating times at 50°

Heating time, min	*Optical density*	
	*NaTD**	*NaTCD**
10	0.449	0.459
15	0.474	0.481
20	0.467	0.480

* Abbreviations: see Table I.

TABLE VII

QUANTITATIVE PAPER CHROMATOGRAPHY OF TAURODEOXYCHOLIC AND TAUROCHENODEOXYCHOLIC ACIDS SINGLY AND IN MIXTURE

*Acid***	*Number of determinations*	*Amount (μg)*	*Recovery** %
TD	2	20	94.7; 92.9
TCD	2	20	104.0; 104.0
TD + TCD	2	20 + 20	98.8; 96.2

* Based on the value of standard samples of TCD.
** Abbreviations: see Table I.

Taurodeoxycholic acid has its main absorption maximum at 389 mμ after being heated in 65% sulphuric acid at 60° for 60 min, whereas taurochenodeoxycholic acid under similar conditions has its main maximum at 305 mμ[17]. This fact was used in an

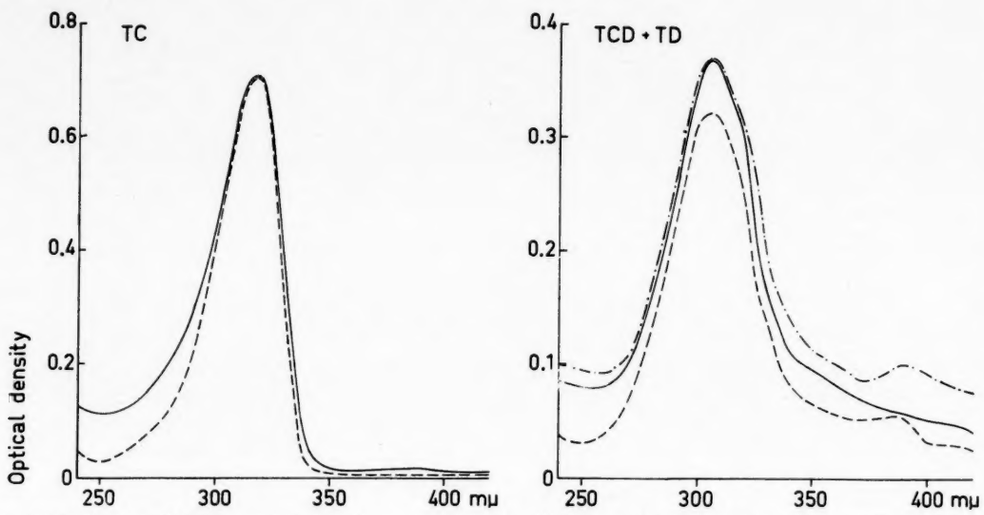

Fig. 3. Sulphuric acid absorption spectra of synthetic bile acids and eluates of the zones corresponding to these acids on chromatograms of duodenal contents. For abbreviations and conditions see Table I. Broken lines: Duodenal contents. Solid lines: Synthetic acids. (·—·—·) in right figure represents the synthetic TD. Spectra of bile acids in duodenal contents are corrected for interfering material (see text).

References p. 664

attempt to determine both acids simultaneously in a mixture. The results, however, were too variable to be of value.

The sulphuric acid spectra of the glycine- and taurine-conjugated bile acids after paper chromatography of duodenal contents are shown in Figs. 3 and 4. The conditions for the sulphuric acid treatment were the same as in the quantitative measurements. The spectra are generally in satisfactory agreement with those obtained with the synthetic bile acids.

A large number of analyses of one bile sample was carried out to investigate the reproducibility of the methods; the results are shown in Table VIII. As shown in Tables IX and X, bile acids added to bile and duodenal contents were recovered in amounts usually between 90 and 110% of the amount added.

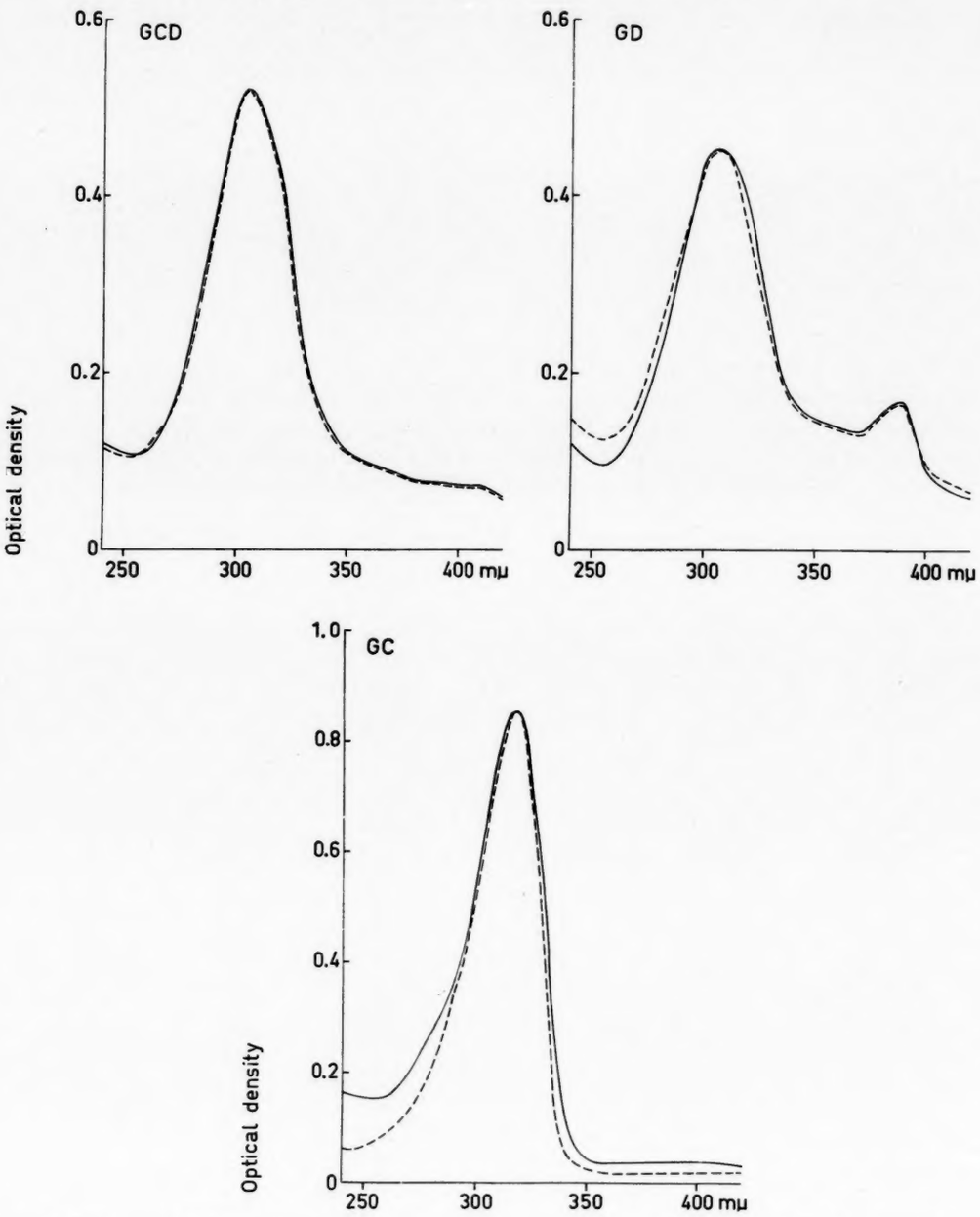

Fig. 4. Sulphuric acid absorption spectra of glycine-conjugated bile acids in duodenal contents. For abbreviations and conditions see Table I. The spectrum of GC from duodenal contents is corrected for interfering material. Broken lines: Duodenal contents. Solid lines: Synthetic acids.

References p. 664

TABLE VIII

REPRODUCIBILITY IN QUANTITATIVE PAPER CHROMATOGRAPHY OF HUMAN BILE

*Bile sample**	*Amount of bile (μl)*	*Number of determinations*	*Amount of bile acid found (μg)*	
			Mean	*Range*
S.H. Diluted gall-bladder bile (10 ×)				
NaTC	20	6	27.9	27.3–28.4
NaTCD + NaTD	20	10	46.0	44.2–47.3
GC	10	16	30.6	27.6–33.5
NaGCD	10	14	26.6	23.9–30.6
GD	10	18	18.1	15.2–20.6
M.N. Fistula bile				
GC	20	6	14.0	13.0–15.0
NaGCD	20	12	6.0	4.7–6.9
GC	40	8	27.2	25.9–28.6
NaGCD	40	6	13.4	11.7–15.0
GC	60	5	38.2	35.5–41.2
NaGCD	60	4	20.5	18.9–21.4
NaTC	40	4	48.1	47.1–49.1
NaTCD	40	4	17.2	16.5–18.0

* Abbreviations: see Table I.

TABLE IX

RECOVERY OF TAURINE-CONJUGATED BILE ACIDS ADDED TO BILE AND DUODENAL CONTENTS

Amounts are given as μg bile salts eluted from the chromatograms. Except for the analyses of bile from S.H., where the values are the mean of two determinations, all values represent single determinations. When present, NaTD is included in NaTCD

Bile sample	*NaTC (μg)**			*NaTCD (μg)**			*Recovery per cent of*	
	added	*calc.*	*found*	*added*	*calc.*	*found*	*added*	*calc.*
S.H.	–	–	27.3	–	–	47.1	–	–
Diluted	5	32.3	32.7	–	–	47.6	108.0	101.2
gallbladder	10	37.3	37.6	–	–	47.6	103.0	100.8
bile (10 ×)	15	42.3	42.6	–	–	46.5	102.0	100.7
20 μl	20	47.3	45.8	–	–	48.5	92.5	96.8
K.N.	–	–	10.4	–	–	18.6	–	–
Diluted	–	–	10.3	–	–	18.1	–	–
gallbladder	10.7	21.1	22.8	–	–	18.5	115.9	108.1
bile (10 ×)	10.7	21.1	22.7	–	–	18.9	115.0	107.6
20 μl	–	–	10.7	10.1	28.5	28.2	97.0	98.9
	–	–	10.7	10.1	28.5	28.3	98.0	99.3
M.N.	–	–	22.1	–	–	8.5	–	–
Fistula bile	–	–	22.2	–	–	8.8	–	–
20 μl	10.9	33.1	33.5	–	–	8.4	103.7	101.2
	10.9	33.1	33.3	–	–	8.3	101.8	100.6
	–	–	22.4	19.7	28.4	27.5	95.4	96.8
	–	–	20.8	19.7	28.4	27.5	95.4	96.8
O.P.	–	–	13.4	–	–	29.4	–	–
Duodenal	–	–	12.2	–	–	30.1	–	–
contents	10.7	23.5	22.7	–	–	31.4	92.5	96.6
40μl	21.4	34.2	34.0	–	–	33.7	99.0	99.4
	–	–	13.7	10.0	39.8	39.2	94.0	98.5
	–	–	14.1	20.0	49.9	52.0	110.4	104.2

* Abbreviations: see Table I.

References p. 664

TABLE X

RECOVERY OF GLYCINE-CONJUGATED BILE ACIDS ADDED TO BILE AND DUODENAL CONTENTS

Amounts are given as μg bile acids or salts eluted from the chromatograms. Values for the fistula bile of M.N. are the mean of two determinations as are also the values for GC from the duodenal contents of J.S. Other values represent single determinations.

Bile sample	*GC (μg)**			*NaGCD (μg)**			*GD (μg)**			*Recovery per cent of*	
	added	*calc.*	*found*	*added*	*calc.*	*found*	*added*	*calc.*	*found*	*added*	*calc.*
S.H.	–	–	29.6	–	–	26.0	–	–	18.0	–	–
Diluted	–	–	29.6	–	–	26.5	–	–	18.2	–	–
gall-	–	–	29.6	10.2	36.5	35.4	–	–	18.7	89.2	97.0
bladder	–	–	29.6	10.2	36.5	36.1	–	–	18.0	96.0	98.9
bile (10 ×)	–	–	29.6	–	–	26.0	10.5	28.6	27.0	84.8	94.4
10 μl	–	–	29.6	–	–	25.6	10.5	28.6	27.2	86.7	95.1
M.N.	–	–	15.0	–	–	6.9			–		
Fistula	3.7	18.7	19.4	–	–	6.6			–	118.9	103.7
bile	7.2	22.2	22.3	–	–	6.0			–	101.4	100.5
20 μl	10.6	25.6	25.9	–	–	6.5			–	102.8	101.2
J.S.	–	–	20.5	–	–	15.9	–	–	11.6	–	–
Duodenal				–	–	16.7	–	–	9.7	–	–
contents	–	–	21.3	10.1	26.4	25.7	–	–	11.8	93.1	97.3
10 μl				10.1	26.4	26.9	–	–	10.3	105.0	101.9
	–	–	20.8	–	–	15.7	10.2	20.9	19.9	90.2	95.2
				–	–	15.9	10.2	20.9	23.6	126.4	112.9

* Abbreviations: see Table I.

In some cases, bile was hydrolyzed (2 *N* NaOH, 6 h, 120°) and the free bile acids were determined after separation with phase system F_d. Within the errors of the method, the proportions between the bile acids were the same as for the conjugated acids, with possibly a minor decrease in the proportion of chenodeoxycholic acid. In some bile samples, the proportions between the glycine-conjugated trihydroxy and dihydroxy acids were determined by reversed phase chromatography, using the phase systems described by NORMAN[18]. The effluent from the column was titrated with 0.02 *N* sodium hydroxide. The values agreed with those obtained by quantitative paper chromatography. No separation of chenodeoxycholic and deoxycholic acids, however, can be obtained with column chromatography, nor is it possible to determine the taurine-conjugated bile acids by this method.

Preliminary results have been published of the analysis of bile acids in normal humans by the present technique[19] and also some data on the excretion of bile acids in choledochostomy drainages[20].

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SUMMARY

A method is described for the determination of bile acids in biological material by quantitative paper chromatography. It has been applied to the analysis of rat, rabbit and human bile and of human duodenal contents.

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IMMUNOELEKTROPHORETISCHE UNTERSUCHUNGEN ÜBER DEN SERUMIDENTISCHEN ANTEIL DER HARNKOLLOIDE UND IHRE BEDEUTUNG BEI DER HARNSTEINBILDUNG

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Unter den zahlreichen Theorien über die Harnsteingenese[1] stehen sich noch heute die Schulen von LICHTWITZ[2, 19] und EBSTEIN[3] gegenüber. Die eine Autorengruppe sieht in den Harnkolloiden eine Schutzfunktion, während die andere annimmt, dass Eiweissubstanzen des pathologischen Harnes sich in die Matrix der Harnsteine umwandeln.

BOYCE, GARVEY UND NORFLEET[4] haben aus den Harnkolloiden eine Mucoproteidfraktion isoliert, welche mit Calcium eine unlösliche chelatartige Verbindung eingeht.

DULCE[5] stellte aus Harnkolloiden und löslichem Kollagen *in vitro* unlösliche Symplexe her, die Calcium und Oxalat einlagerten. Er nimmt an, dass es zu Beginn der Steingenese zu einer Symplexbildung aus zwei oder mehreren Eiweissen oder Mucoproteiden kommt, wobei eine der Substanzen nur im Harn von Steinkranken auftritt. Als derartige Symplexbildner können auch die von KOCH und Mitarb.[6, 7] bei der experimentellen Harnsteinkrise aufgefundenen Kolloidkörperchen infrage kommen, denn es gelang HARADA und SAITO[8] in diesen Kolloidkörperchen Mucoproteide färberisch nachzuweisen.

Primär entsteht also die organische Steinmatrix aus Harnkolloiden oder bestimmten Fraktionen der Kolloide, die zum Harnstein "verkalken". Danach müssten enge Beziehungen zwischen den Harnkolloiden oder den Symplexen und der Steinmatrix zu erwarten sein. Diesbezügliche Untersuchungen wurden nach entsprechender Aufarbeitung und Hydrolyse von Harnkolloiden und Mucoproteiden aus dem Harn Gesunder und Steinkranker einerseits und der Steinmatrix andererseits mit Hilfe papierchromatographischer Bausteinanalysen der Aminosäuren und Kohlehydrate durchgeführt[5, 9, 10].

Die Unterschiede in den Aufarbeitungsverfahren, die eine Zerstörung der Eiweissstruktur bewirken, und die Tatsache, dass die Steinmatrix und die Harnkolloide ein Gemisch zahlreicher Eiweissfraktionen darstellen, führen zu Schwierigkeiten in der Beurteilung derartiger Bausteinanalysen[11].

Um die Eiweisse im Harnkolloid weitgehend zu schonen, wurde ein neuer Weg mit Hilfe der Elektrophorese gesucht. PEDERSEN[12] konnte dabei im Urin Albumin, γ-Globulin, wenig α-Globulin und β-Globulin mit einem Molekulargewicht unter 150,000 nachweisen, während die grossmolekularen Lipoproteine nicht in den Urin übertreten sollen. Auch WUNDERLY UND CASPANI[13] gelang im angereicherten Uroprotein von Gesunden keine Lipoidanfärbung.

Bei der Makroelektrophorese stimmten die Pherogramme von Uroproteinen und Blutserum erstaunlich gut überein[14]. Allerdings bestand ein quantitativer Unterschied: der Albumin–Globulin-Quotient der Uroproteine lag im Durchschnitt bei 0.51, der des Serums bei 1.72.

Literatur S. 673

Um bei der Analyse der Harnkolloide weiterzukommen, haben wir erstmalig folgende Einteilung versucht:

1. Gruppe A umfasst die Harnkolloide, die mit Serumeiweissen und Serummucoiden identisch sind,
2. Gruppe B umfasst diejenigen Eiweisse, Mucoproteide oder anderen kolloidalen Substanzen, die nur im Harn vorkommen.

Als sicherer Identitätsnachweis der Harnkolloide mit dem Serumeiweiss ist die Immunoelektrophorese nach GRABAR UND WILLIAMS[15] geeignet. Sie ermöglicht nach elektrophoretischer Trennung die Charakterisierung der Eiweisse auf Grund ihrer immunologischen Eigenschaften. Bei Verwendung eines Antihumanserums (AHS)* werden nur die serumidentischen Anteile der Harnkolloideiweisse erfasst, während über das Vorhandensein nicht-serumidentischer Proteine im Harnkolloid bei dieser Versuchsanordnung nichts ausgesagt werden kann. Selbst für den Fall, dass der serumidentische Anteil der Harnkolloide bei der Harnsteinbildung keine unmittelbare Rolle spielt, ist mit den Versuchen doch die Möglichkeit einer verfeinerten Analyse und evtl. einer weiteren Isolierung einzelner Fraktionen aus den Harnkolloiden und der Steinmatrix aufgezeigt.

Wir untersuchten nach entsprechender Anreicherung der Harnkolloide den serumidentischen Anteil im Harn von Gesunden und Steinkranken mit der Immunoelektrophorese. Eine Kontrolle dieser Ergebnisse wurde durch analoge Absättigungsversuche durchgeführt. Um die angenommenen Beziehungen zwischen Harnkolloiden und Steinmatrix näher zu erfassen, wurden auch Absättigungsversuche mit fein vermahlenem Harnsteinpulver und dem AHS angesetzt.

METHODIK

Anreicherung der Harnkolloide

Der 24-Stunden-Sammelharn von Gesunden und Urolithiasiskranken mit Oxalatsteinanamnese wurde nach scharfem Abzentrifugieren der korpuskulären Bestandteile im Ultrafiltrationsgerät von 200 ml Fassungsvermögen durch allerfeinste Filter (fff, Membranfilter-Gesellschaft Göttingen) filtriert. Eine Dialyse führten wir nicht durch, da bekannterweise ein Kohlenhydratanteil dabei verloren geht. Der bräunlich gefärbte Rückstand der Harnkolloide wurde vom Filter abgenommen und im evakuierten Exsiccator über $CaCl_2$ getrocknet, anschliessend im Mörser fein zerrieben. Ein Teil der auf diese Weise gewonnenen Harnkolloide kam zur chemischen Analyse, ein weiterer Anteil wurde immunoelektrophoretisch getestet.

Bei einigen Parallelversuchen reicherten wir die Harnkolloide durch Acetonfällung an. Dazu wurde der Sammelharn nach Vorkühlung (+4°) im Verhältnis 1:1 mit Aceton versetzt und nach 10–14 Stunden Aufbewahrung im Eisschrank zentrifugiert. Nach Trocknung des Sedimentes—wie oben beschrieben—erfolgte die gleiche Weiterverarbeitung.

Da die immunoelektrophoretischen Ergebnisse übereinstimmen, werden sie gemeinsam ohne weitere Unterscheidungen besprochen.

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* Antisérum humain total du cheval (Institut Pasteur, Paris).

Literatur S. 673

Chemische Analyse

In Doppelversuchen wurden 10 mg der jeweils gewonnenen Harnkolloide (Frischsubstanz) 24 Stunden im Trockenschrank bei 105° getrocknet (= Trockensubstanz). Der Gewichtsverlust entsprach dem Wassergehalt. Der anorganische Anteil der Harnkolloide wurde durch Auswiegen des Glührückstandes ermittelt. Der organische Anteil ergab sich aus der Differenz der beiden Wägungen. Den Eiweissgehalt des organischen Anteils in der Frischsubstanz bestimmten wir mit der Biuretprobe und berechneten ihn. Die Differenz zwischen dem Eiweissanteil und der gesamten organischen Substanz entsprach dem übrigen organischen Anteil.

Immunoelektrophorese

Für die immunoelektrophoretischen Untersuchungen wandten wir die Originalmethode von GRABAR UND WILLIAMS[15] an. Bei jeder Immunoelektrophorese lief zum Vergleich ein Humanserum mit, wobei gleiche Mengen Eiweiss von Harnkolloiden und Serumeiweiss aufgetragen wurden. Die Harnkolloid-Frischsubstanz lösten wir in einer jeweils entsprechenden Menge physiologischer Kochsalzlösung, so dass die aufgetragene absolute Eiweissmenge entsprechend der Berechnung in allen Fällen 1.4 mg betrug. Nach elektrophoretischer Trennung bei 120 V und 50–70 mA wurden die Präcipitationsreaktionen mit Hilfe eines mit physiologischer Kochsalzlösung 1:4 verdünnten AHS durchgeführt. Bei einer solchen Auswertung müssen alle im Harnkolloid enthaltenen serumidentischen Eiweisskomponenten reagieren.

Absättigungsversuche

Eine weitere Kontrolle der immunoelektrophoretischen Ergebnisse erfolgte durch Absättigung eines AHS mit Harnkolloiden von Normalen und Steinkranken. Zu diesem Zwecke wurde 1 ml eines 1:4 mit physiologischer Kochsalzlösung verdünnten AHS mit der 15-fachen Menge der bei der direkten Immonoelektrophorese aufgetragenen Harnkolloide versetzt. Das Gemisch blieb 4 Stunden bei Zimmertemperatur stehen und wurde dann 10 Minuten bei 4,000 U.p.M. zentrifugiert zur Beseitigung aller infolge einer Antigen–Antikörper-Reaktion zwischen den serumidentischen Harnkolloideiweissen und ihren entsprechenden Antikörpern gebildeten Präcipitate. Bei der folgenden Auswertung des abgesättigten AHS gegen ein Normalserum müssen also alle diejenigen Präcipitatlinien fehlen, deren zugehörige Antikörper bereits abgebunden wurden.

Die Ringreaktionen der direkten Immunoelektrophorese und diejenigen des Absättigungsversuches müssen sich zur Summe der Präcipitationen ergänzen, die bei Auswertung des AHS gegen ein Normalserum auftreten.

Absättigungsversuche mit Steinmatrix von Oxalatsteinen

Eine Herauslösung der Eiweisskörper aus der Steinmatrix, bzw. die Entfernung der anorganischen Bestandteile durch Säuren erschien uns problematisch, weshalb eine direkte immunoelektrophoretische Untersuchung der Steinmatrixkolloide bis jetzt nicht versucht wurde. Falls aber in die Steinmatrix serumidentische Eiweisskörper aus den Harnkolloiden mit eingebaut waren, die bei der Steinbildung ihre serologische Reaktionsfähigkeit nicht eingebüsst hatten, musste ein Absättigungsversuch durchführbar sein. Bei diesem Vorgehen waren Veränderungen an den Eiweissmolekülen durch vorausgehende Bearbeitung nicht zu erwarten.

Literatur S. 673

Wir verwandten feinstes Pulver von operativ entfernten Oxalatsteinen nach 24-stündigem Trocknen über $CaCl_2$ im Exsiccator. 1.5 g Harnsteinpulver vermischten wir unter ständigem Rühren mit 0.6 ml AHS und 1.8 ml physiologischer Kochsalzlösung. Das Gemisch wurde 6 Tage im Eisschrank aufbewahrt und regelmässig umgerührt. Nach Abzentrifugieren testeten wir 1 ml des überstehenden abgesättigten AHS gegen Normalserum wie oben beschrieben.

ERGEBNISSE

Die Ergebnisse der chemischen Analyse angereicherter Harnkolloide sind in Tabelle I zusammengestellt.

TABELLE I

CHEMISCHE ANALYSE DER IMMUNOELEKTROPHORETISCH UNTERSUCHTEN HARNKOLLOIDE BEI GESUNDEN UND STEINKRANKEN

	Gesamtharn-menge	*Trockensubstanz*	*Anorganiser Substanzanteil*	*Organischer Substanzanteil*	
				Proteine	*Nichtproteine*
	ml	*mg*	%	%	%
Normalharn:					
1. D.	1500	235.6	19.2	54.4	26.4
2. B.	2000	400.1	11.3	32.6	56.1
3. K.	1500	277.5	23.2	41.5	35.3
4. H.	1850	265.4	22.1	35.8	42.1
5. Kr.	1450	270.5	20.1	41.1	38.8
Durchschnittlich	**auf 1000 ml:**	174.6 mg	33.5 mg	71.6 mg	69.4 mg
Steinharn:					
1. Ka.	1860	252.2	11.9	70.5	17.6
2. M.	2000	546.0	34.1	25.1	30.8
3. W.	2115	273.0	10.9	50.7	38.4
4. Mü.	2000	250.5	11.3	84.4	4.3
5. Mo.	1900	232.0	13.7	57.3	29.0
Durchschnittlich	**auf 1000 ml:**	157.3 mg	25.8 mg	90.6 mg	40.9 mg

Beim Gesunden wurden im Mittel 174.6 mg Trockensubstanz bezogen auf 1 Liter Harn gewonnen. Bei den Steinkranken lag der Wert bei 157.3 mg. Der Proteinanteil errechnete sich im Durchschnitt zu 71.6 mg bei Gesunden und 90.6 mg bei Steinkranken. Diese an je 5 Fällen ermittelten Werte zeigen keine signifikanten Differenzen in den beiden Versuchsgruppen. Die Voranalyse diente lediglich dazu, Anhaltspunkte über die Zusammensetzung des zur Immunoelektrophorese benützten Materials zu gewinnen. Dadurch war es auch möglich, Harnkolloide und Serum in etwa gleichen Eiweiss-Konzentrationsverhältnissen zu untersuchen.

Die Ergebnisse der Immunoelektrophorese sind in der Fig. 1 zusammengestellt. Bei unseren Versuchen hat das AHS (Institut Pasteur, Paris) folgende Präcipitationsringe im Normalserum (Fig. 1,1) ergeben:

1 Albuminlinie, 1 x-Linie*, 1 α_1-Linie, 4–6 α_2-Linien, davon 1 α_2-Makro- und 1 α_2-Lipoproteinlinie; 3 β_1-Linien, davon 1 Siderophylinlinie; 3 β_2-Linien, davon 1 β_2-Makroglobulinlinie; 1 γ-Linie.

* Anmerkung: Die x-Fraktion wird neuerdings von DE VAUX ST.-CYR, COURCON UND GRABAR[16] mit "α^1-Seromucoide acide" bezeichnet.

Literatur S.673

Diese Linien entsprechen den im allgemeinen auftretenden 14–16 Präcipitationslinien des von uns verwandten Antihumanserums.

In den Harnkolloiden gesunder Personen (Fig. 1,2) fanden sich insgesamt 6–7 serumidentische Komponenten:

1 Albuminlinie, 1 x-Komponente, 1 α_1-Linie, 2 α_2-Linien. 1 β_1-Linie und 1 γ-Linie.

Alle Präcipitationslinien wurden annähernd konstant gefunden mit Ausnahme der γ-Linie. Auf die Inkonstanz dieser Linie wird in der Diskussion noch kurz eingegangen.

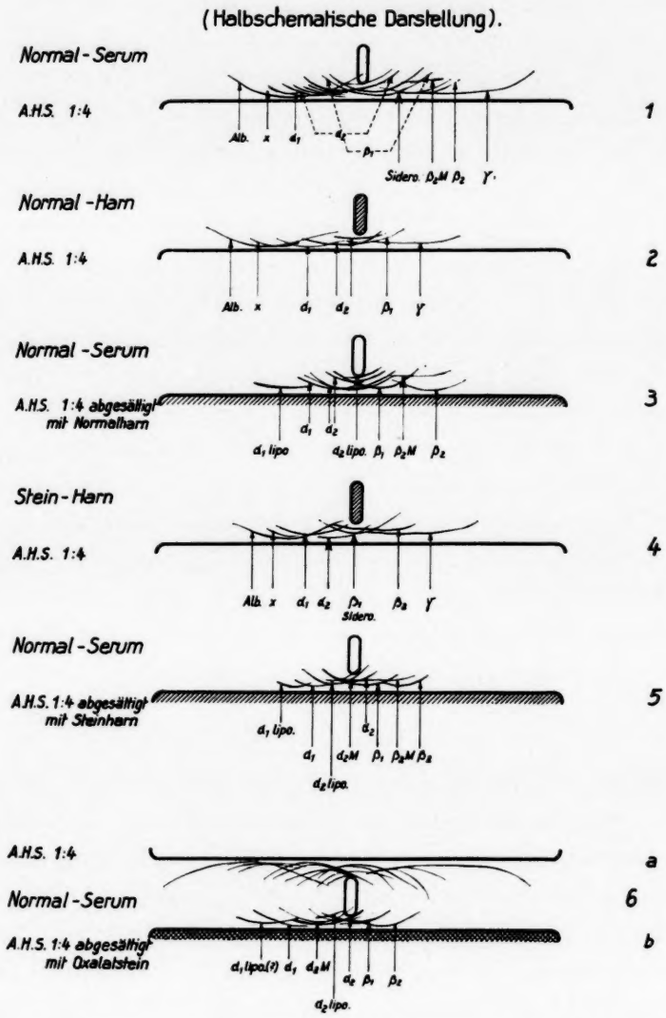

Fig. 1. Vergleich des immunoelektrophoretischen Verhaltens von Serum, Harnkolloiden von Normalen und Urolithiasiskranken, sowie von Harnsteinen.

Im entsprechenden Absättigungsversuch (Fig. 1,3) fehlten erwartungsgemäss alle diejenigen Linien, die bei der Immunoelektrophorese ausgefallen waren, besonders konstant das γ-Globulin, d.h. es ist bei einer Versuchsanordnung mit überschüssiger Antigenmenge im Harn nachweisbar.

Die Untersuchungen der Harnkolloide von Steinkranken (Fig. 1,4) ergaben folgende 7–8 serumidentische Komponenten:

1 Albuminlinie, 1 x-Komponente, 1 α_1-Linie, 2 α_2-Linien, 1 β_1-Linie, 1 β_2-Linie, 1 γ-Linie.

Literatur S. 673

Eine geringe Inkonstanz zeigten die α_2-Linien. Die γ-Linie ist hier im Gegensatz zu den Harnkolloiden Gesunder ganz konstant aufgetreten. Auch die übrigen Linien waren stets konstant reproduzierbar. 1 β_2-Linie ist mit nur einer Ausnahme regelmässig nachweisbar gewesen.

Nach den Versuchen enthält der Harn von Steinkranken wahrscheinlich eine β_2-Komponente, die von uns im Normalharn im Gegensatz zu PATTE, BALDASSAIRE UND LORET[17] nicht gefunden wurde. Die erwähnte β_1-Fraktion scheint mit dem Siderophylin identisch zu sein.

Der Absättigungsversuch fiel auch hier im wesentlichen übereinstimmend mit der direkten Immunoelektrophorese aus (Fig. 1,5).

Bei Absättigung des AHS mit Oxalatsteinpulver und nachfolgender Auswertung gegen ein Humanserum sind nur 7 Linien ausgefallen (Fig. 1,6). Die folgenden 7–9 Komponenten fehlen: die Albuminlinie, die x-Linie, die α_1-Linie erscheint abgeschwächt, 2 α_2-Linien, 1 β_1-Linie, die dem Siderophylin entsprechen dürfte, 1 weitere β_1-Linie ist abgeschwächt. Ausserdem fehlen 1 β_2-Linie und die γ-Linie.

Nach dem Absättigungsversuch müssten also die aufgezählten fehlenden Eiweisskomponenten in der Steinmatrix vorhanden sein. Die Ergebnisse fielen bei mehreren Versuchen mit Ausnahme der x-Linie (Séromucoïde acide) konstant aus.

Weniger sichere Angaben lassen sich über die α_1- und β_1-Linien machen, da diese nicht gänzlich fehlten. Es scheint jedoch durchaus möglich, dass auch diese beiden Komponenten in der Steinmatrix vorhanden sind, allerdings mengenmässig zu einer völligen Absättigung nicht ausreichen.

Setzt man die Ergebnisse der immunoelektrophoretischen Untersuchungen der Harnkolloide von Normalen und von Harnsteinkranken in Beziehung, so ergibt sich, dass *in beiden Fällen die gleichen serumidentischen Eiweisse vorkommen.* Auch der Vergleich zwischen Harnsteinkolloiden und Steinmatrix zeigt *bei Oxalatsteinbildnern dieselben Eiweisskomponenten in Matrix und Harnkolloiden.*

DISKUSSION

Unsere Ergebnisse bei Normalurinen decken sich weitgehend mit den vor kurzer Zeit erschienenen Untersuchungen von GRANT[18], PATTE und Mitarb.[17]. Allerdings fanden wir bei der direkten Immunoelektrophorese nicht immer das γ-Globulin. Im Gegensatz dazu war γ-Globulin nach den Absättigungsversuchen im Harn Normaler stets vorhanden. Danach nehmen wir in Übereinstimmung mit GRANT und PATTE an, dass γ-Globuline zum normalen Harnkolloid gehören und führen unsere inkonstanten Ergebnisse auf ein ungünstiges Antigen–Antikörperverhältnis zurück, da wir ja—wie oben erwähnt—zur besseren Vergleichbarkeit von stets gleichen Eiweissauftragungsmengen ausgingen. Wahrscheinlich ist beim Normalharn nicht genügend γ-Globulin durch die direkte Immunoelektrophorese getrennt worden, während beim Absättigungsversuch die γ-Globulinmenge zum Nachweis ausreicht. Bei den übrigen Fraktionen fanden wir keine Unterschiede.

Der Urin von Harnsteinkranken wies die gleichen Eiweisskomponenten wie Normalharn auf. Ausserdem enthielt er noch eine β_2-Komponente, die wir auch nach Anreicherung bei Gesunden nicht nachweisen konnten. Diese Diskrepanz der Befunde von PATTE[17] einerseits und denjenigen von GRANT[18] und uns andererseits beruht möglicherweise auf dem qualifizierteren Antihumanserum von PATTE, das 24 Fraktionen gegen Normalserum enthielt.

Literatur S. 673.

Die Lipoproteine und das α_2- und β_2-Makroglobulin treten im Harn von Gesunden und Steinkranken nicht auf. Daraus folgt, dass beim Vorhandensein dieser Fraktionen im Harn wahrscheinlich eine Beimengung von Blut, bzw. Serum vorliegt.

Die x-Fraktion ist im Normalharn und auch im Steinharn nachweisbar.

Aus den Absättigungsversuchen mit Oxalatsteinpulver glauben wir folgern zu können, dass die Steinmatrix einen Teil serumidentischer Eiweisskomponenten enthält. Da die gleichen Komponenten auch im Harn von Steinkranken vorkommen, darf angenommen werden, dass diese Eiweisse aus den Harnkolloiden in die Steinmatrix eingebaut werden.

Wie zu erwarten war, sind serumidentische Eiweisse, die im Harnkolloid fehlen, auch in der Steinmatrix nicht nachzuweisen. Obwohl wir die Möglichkeit unspezifischer Adsorptionen von Antikörpern an das Harnsteinpulver in Betracht zogen, glauben wir, dass eine solche Bindung nicht vorliegt, da die immunoelektrophoretischen Ergebnisse von Steinkranken-Harnkolloiden und Steinmatrix auffallend gut übereinstimmen. Es soll dazu kurz erwähnt werden, dass die Absättigungsversuche mit verschiedenartigen Harnsteinen unterschiedlich ausgefallen sind, was unseres Erachtens nach ebenfalls gegen unspezifische Veränderungen spricht.

Die Theorien über die Herkunft der Harnkolloide weichen stark voneinander ab. LICHTWITZ[19] nahm an, dass alle Harnkolloide aus dem Blut stammen; BAKER UND CONELLY[20] folgerten dagegen, dass die Harnkolloide von den Nierentubuli sezerniert werden. Nach den vorliegenden Ergebnissen glauben wir, dass wohl nur der serumidentische Anteil der Harnkolloide direkt aus dem Blut bzw. Serum durch die Glomerulusmembran in den Harn übertritt. Die grossmolekularen Serumeiweisskomponenten und die Lipoproteine werden vollständig zurückgehalten. Gleiche Schlussfolgerungen ziehen auch GRANT und PATTE.

Die nicht-serumidentischen Eiweisse der Harnkolloide werden von den Tubulusepithelien sezerniert[21]. Eine direkte Herkunft aus dem Blutserum ist unwahrscheinlich.

Sicherlich sind die einzelnen Anteile der Harnkolloide ganz unterschiedlicher Abstammung, wodurch die *Einteilung in einen serumidentischen und einen nicht-serumidentischen Anteil gerechtfertigt erscheint.*

DANK

Wir danken Herrn Professor P. GRABAR, Paris, Institut Pasteur, für seine beratende Unterstützung.

Diese Arbeit wurde mit Hilfe der Deutschen Forschungsgemeinschaft durchgeführt.

ZUSAMMENFASSUNG

In der Einleitung wird ein kurzer Überblick über den Stand und die Problematik der Harnsteingenese gegeben und begründet, warum sich die Immunoelektrophorese zur näheren Untersuchung des "serumidentischen Anteiles" der Harnkolloide eignet.

Wir konnten nachweisen, dass ein gewisser Anteil der Harnkolloide aus Eiweissen besteht, die nach ihrer Wanderungsgeschwindigkeit und nach ihren serologisch aktiven Gruppen mit Serumeiweissen übereinstimmen. Im normalen Harnkolloid

Literatur S. 673

fanden sich Albumin, eine x-Fraktion ("séromucoïde acide"), 1 α_1-Globulin, 2 α_2-Globuline, 1 β_1-Globulin und γ-Globulin. α_1-Lipo-, α_2-Lipo-, α_2-Makro- und β_2-Makro-Globulin kommen im Harn nicht vor.

Das Harnkolloid von Normalen und Oxalatsteinkranken zeigte praktisch die gleichen serumidentischen Eiweisskomponenten. Absättigungsversuche und direkte Immunoelektrophorese fielen weitgehend übereinstimmend aus.

Auf den direkten Übertritt von Serum oder Blut in die ableitenden Harnwege lässt sich dann schliessen, wenn die normalerweise im Harnkolloid nicht vorkommenden Eiweisskomponenten wie die Lipo- und Makroglobuline nachgewiesen werden können.

In die Steinmatrix von Oxalatsteinen werden praktisch alle im Harnkolloid vorhandenen Serumeiweisskomponenten mit eingebaut. Serumeiweisse, die normalerweise im Harn nicht vorkommen, konnten auch in der Steinmatrix nicht nachgewiesen werden.

Sehr wahrscheinlich passiert der serumidentische Anteil der Harnkolloide die Glomerulusmembran und gelangt so aus dem Serum in den Urin. Grossmolekulare Eiweisse werden dagegen vollständig zurückgehalten.

SUMMARY

IMMUNO-ELECTROPHORETIC INVESTIGATION OF THE URINARY COLLOIDS IDENTICAL WITH SERUM COLLOIDS AND THEIR SIGNIFICANCE FOR THE FORMATION OF URINARY CALCULI

The origination of urinary calculi and the problems connected therewith are briefly discussed.

Immuno-electrophoresis was found to be a suitable method for studying the urinary colloids. It was found that a certain fraction of the urinary colloids consists of proteins that are identical with serum proteins as regards migration velocities and serologically active groups. The following proteins are present in normal urinary colloids: albumin, an x-fraction ("séromucoïde acide"), one α_1-globulin, two α_2-globulins, one β_1-globulin and one γ-globulin. α_1- and α_2-lipoglobulins and α_2- and β_2-macroglobulins do not occur in urine.

There was scarcely any difference between the urinary colloids of normal persons and those of patients suffering from oxalate calculi, as regards those components that also occur in serum. The results obtained in saturation experiments and direct electrophoresis were practically identical.

If protein components that are not normally present (such as lipoglobulins and macroglobulins) are found in urinary colloids, it can be concluded that serum or blood has passed directly into the urinary ducts.

Practically all the serum protein components that occur in urinary colloids are also found in the stone matrix of oxalate calculi. Serum proteins not normally present in urine could not be detected in the matrix.

The authors consider it very likely that the presence of these serum proteins in the urine is due to their ability to pass through the glomerulus membrane. Macroglobulins, on the other hand, are unable to pass through this membrane.

Literatur S. 673

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LIVER FUNCTION AND AGE

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INTRODUCTION

In the previous article[1] intravenous loading tests of galactose and bromsulfalein were given. From their disappearance curves the total capacity of the liver for these substances was deduced and expressed by the concept of "half-life" ($t_{\frac{1}{2}}$).

The purpose of the present article is to determine the normal values of $t_{\frac{1}{2}}$ for galactose and bromsulfalein. As $t_{\frac{1}{2}}$ appeared to depend on age, this relationship is discussed and analysed. As a result, age-"independent" standards in these functional tests are given.

PROCEDURE

A. Selection of cases and methods

The determination of $t_{\frac{1}{2}}$ of galactose and bromsulfalein was carried out in individuals (mainly patients) in whom both clinically and on the basis of laboratory tests, liver disease was improbable. In the patients this was tested by the serum bilirubin, alkaline phosphatase, urobilin in the urine, thymol and Gros' turbidity tests. None of them had fever. Patients with kidney disease and disturbances in growth and in circulation were excluded from this group. The galactose group consisted of 90, and the bromsulfalein group of 45 individuals.

Both the galactose (0.5 g/kg) and bromsulfalein (5 mg/kg) loadings were based on body weight. The half-life of both substances was determined graphically[1].

B. Half-life in relation to age in the control groups

It is evident from Figs. 1 and 2, that both in the galactose and bromsulfalein group, $t_{\frac{1}{2}}$ in children is considerably dependent on age.

The number of observations in adults may have been too small to ascertain a slight age-dependency in that age-range.

There is no clear difference between the sexes either for galactose or for bromsulfalein. It is a known rule that in the administration of drugs, test substances, food, etc., children generally get a relatively higher quantity than adults. The investigation of CHOMET[2] is an example of this rule; this author recommends, on the empirical basis of the renal galactose excretion, 30 g galactose in BAUER'S oral test[3] in children weighing between 16 and 30 kg.

BAUER used 40 g galactose in adults. It is thus evident that in children the relative loading is considerably higher than in adults, which may argue for a higher rate of elimination in children.

The cause of the influence of age on $t_{\frac{1}{2}}$ during life is undoubtedly complex. Fac-

* Head: Prof. Dr. G. M. H. VEENEKLAAS.

References p. 682

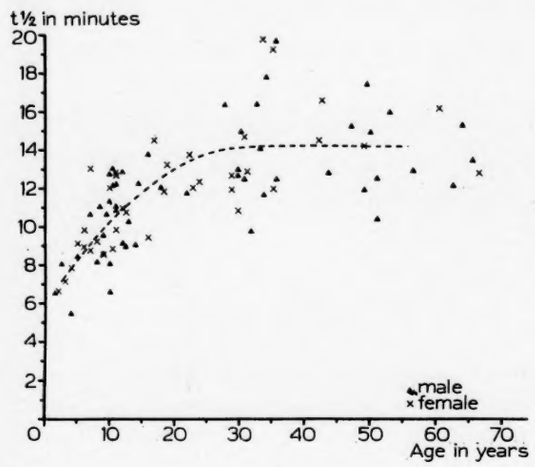

Fig. 1. The relation between age and $t_{\frac{1}{2}}$ in 90 controls for galactose.

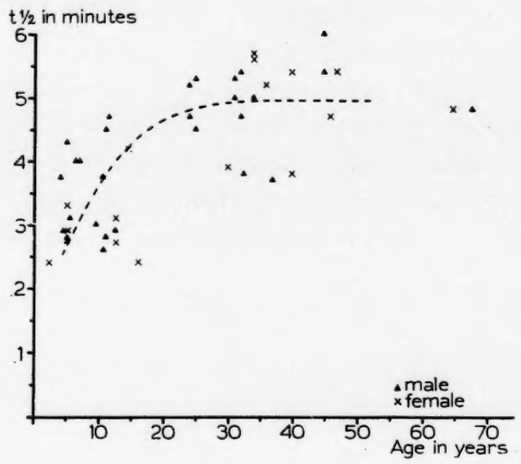

Fig. 2. The relation between age and $t_{\frac{1}{2}}$ in 45 controls for bromsulfalein.

tors such as specific enzymic activity of liver tissue, glucose metabolism[4], relative liver weight, hepatic blood flow, cardiac output, maturity of the liver, basal metabolic rate, circulation time, extent of distribution volume of galactose, etc., would have to be considered.

A higher consumption of milk with an enzymic adaptation to galactose in infants and children is possibly a cause. We found, however, an analogous influence of age on the elimination of bromsulfalein, a substance which is totally different from galactose in its chemical and physiological properties.

Thus, it may be concluded that the influence of age on the rate of elimination is mainly based on a general physiological behaviour, and is not due to the properties of galactose or of bromsulfalein as such.

Although the explanation of the age-dependence must be complex, 5 factors which may be related to the rate of elimination of galactose and bromsulfalein should be discussed.

1. *In vitro* studies of liver homogenates from normal rats have shown that the *enzyme activity* (based on weight) can vary at different ages. In 25 enzymes studied, the activity of nearly all was found to increase with age (see *e.g.*[5]). The activities of enzymes concerned in the elimination of galactose and of bromsulfalein, have not been studied in relation to age. It is not probable, however, that a change of enzyme activity in the elimination of galactose and bromsulfalein could cause the dependence on age found in this study, for the age-dependences in both tests parallel each other and appear to be opposite to those found in most of the other enzymes of rat liver tissue.

2. It is possible that the *relative liver weight* (the liver weight per kg body weight) is related to the age-dependence of $t_{\frac{1}{2}}$. It is therefore of interest to know the change in relative weight of the liver during life. In Fig. 3 this physiological change in men is summarized, as calculated from several studies[7]. From this change it can be concluded that the age-dependent rate in the elimination of galactose and bromsulfalein may be partially caused by a change in the relative liver mass.

3. It is known that the *basal metabolic rate* parallels many cellular and circulatory activities. As the relative need of energy in children is higher than in adults, the basal

References p. 682

metabolic rate may be related to the rate of disappearance of many substances. This would not point, however, to a causal relationship but, for instance, to an energetic or circulatory connection. The investigations of DONE *et al.*[6] and of BROWN *et al.*[8] support this hypothesis. DONE *et al.* found that the rate of removal of cortisol from the plasma and the metabolic rate are faster in children than in adults. BROWN *et al.* state that in hyperthyroidism the rate of removal of cortisol from the plasma is increased, and in hypothyroidism it is decreased.

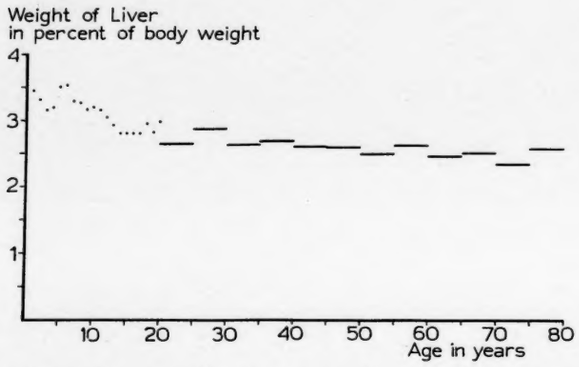

Fig. 3. The relative weight of the human liver during life.

4. It is known in physiology as well as in pathology that the *blood flow of the liver* (portal and arterial) affects the rate of hepatic elimination of several substances (*e.g.*[9]). A quantitative relation, however, is lacking. It is possible, that in infants and children, in addition to a higher basal metabolic rate and blood-circulation rate, the relative hepatic blood flow is higher than in adults.

5. It is improbable that the age-dependence of the *volume of distribution* (V) of galactose[1] can cause the age-dependence of $t_{\frac{1}{2}}$.

a) The age-dependence of $t_{\frac{1}{2}}$ was found both for galactose and bromsulfalein whereas the galactose space was about 4 times the bromsulfalein space.

b) The volume of distribution of bromsulfalein is probably independent of age[1].

Summarizing, it may be said that the age-dependence found in $t_{\frac{1}{2}}$ may be partially related to the basal metabolic rate, and to the gradually decreasing relative liver weight and hepatic blood flow during life.

C. Attempts to eliminate the age-dependence of $t_{\frac{1}{2}}$

We tried to eliminate the age-dependence in order to investigate this physiological phenomenon more closely and to facilitate the interpretation of these tests in health and in disease.

As the basal metabolic rate (BM) appeared to be related to the elimination rate of cortisol (see under B), and as a higher rate of elimination was found in children than in adults, BM was used in our correlation analysis. For this purpose we analysed the relation between age and $t_{\frac{1}{2}} \times$ standard basal metabolic rate (BM_s) and age and $t_{\frac{1}{2}}$/theoretical basal metabolic rate (BM_{th}).

Moreover, we studied the correlation between age and some easily obtainable factors such as $t_{\frac{1}{2}}$/body-weight (BW), $t_{\frac{1}{2}}$/body-length (L), $t_{\frac{1}{2}}$/surface area (SA) and $t_{\frac{1}{2}} \times$ distribution volume (V).

In these relations BM_{th} means the theoretical basal metabolic rate defined by

References p. 682

the age, sex and surface area (SA) of the individual. It was found by the following equation:

$$BM_{th} = \frac{BM \cdot SA}{60} \text{ Cal} \cdot \text{min}^{-1},$$

in which BM should be divided by 60 to convert hours to minutes.

$$BM_s = \frac{BM}{60} \text{ Cal} \cdot \text{m}^{-2} \cdot \text{min}^{-1}$$

As the basis for BM, the normal standards of basal metabolic rate summarized by FLEISCH[10] were used; these are based on 24 reports in the literature. V is the volume of distribution expressed in percentage of body-weight[1]. The SA was deduced from body-length and -weight with nomograms[11, 12], based on the formula of DUBOIS AND DUBOIS[13]. The dimensions of the linearly related derivations of $t_{\frac{1}{2}}$ are:

$$\frac{t_{\frac{1}{2}}}{BW} : \text{min} \cdot \text{kg}^{-1}; \frac{t_{\frac{1}{2}}}{L} : \text{min} \cdot \text{m}^{-1}; \frac{t_{\frac{1}{2}}}{SA} : \text{min} \cdot \text{m}^{-2};$$

$$\frac{t_{\frac{1}{2}}}{BM_{th}} : \text{min}^2 \cdot \text{Cal}^{-1}; t_{\frac{1}{2}} \times V : \text{min}; t_{\frac{1}{2}} \cdot BM_s = \text{Cal} \cdot \text{m}^{-2}.$$

As a first approach to the elimination of the age-dependence in the control groups of galactose and bromsulfalein, the mean values of the above-mentioned factors for some arbitrarily divided age groups, and their standard deviations, were calculated. In these calculations a normal distribution was assumed.

It appeared that both in the galactose and bromsulfalein tests especially $t_{\frac{1}{2}}/L$, $t_{\frac{1}{2}}/BM_{th}$ and $t_{\frac{1}{2}} \cdot BM_s$ give results which are only slightly dependent on age (Tables I and II).

TABLE I

MEAN VALUES (M) OF $t_{\frac{1}{2}}$, $t_{\frac{1}{2}}/L$, $t_{\frac{1}{2}}/BM_{th}$ AND $t_{\frac{1}{2}} \cdot BM_s$ AND THEIR STANDARD DEVIATIONS (σ in %) IN THE CONTROL GROUP OF GALACTOSE

Age group years	*Number of controls*	$t_{\frac{1}{2}}$		$t_{\frac{1}{2}}/L$		$t_{\frac{1}{2}}/BM_{th}$		$t_{\frac{1}{2}} \cdot BM_s$	
		M	σ *in* %	M	σ *in* %	M	σ *in* %	M	σ *in* %
1– 8	11	7.96	18.3	7.81	13.6	14.6	11.7	6.98	10.1
8–15	32	10.6	15.3	7.71	13.6	13.45	14.9	7.70	15.4
15–22	6	12.4	14.5	7.77	10.4	13.8	12.7	7.81	16.6
22–46	28	13.9	18.1	8.32	16.5	13.9	16.1	8.36	19.4
>46	13	13.9	14.3	8.28	15.7	13.7	12.1	7.98	15.7

TABLE II

MEAN VALUES (M) OF $t_{\frac{1}{2}}$, $t_{\frac{1}{2}}/L$, $t_{\frac{1}{2}}/BM_{th}$ AND $t_{\frac{1}{2}} \cdot BM_s$ AND THEIR STANDARD DEVIATIONS (σ in %) IN THE CONTROL GROUP OF BROMSULFALEIN

Age group years	*Number of controls*	$t_{\frac{1}{2}}$		$t_{\frac{1}{2}}/L$		$t_{\frac{1}{2}}/BM_{th}$		$t_{\frac{1}{2}} \cdot BM_s$	
		M	σ *in* %	M	σ *in* %	M	σ *in* %	M	σ *in* %
1– 8	11	3.29	19.0	2.90	13.4	5.20	13.3	2.73	19.1
8–16	11	3.33	24.4	2.36	22.2	4.27	23.0	2.37	22.0
22–40	15	4.87	13.2	2.82	15.8	4.43	19.6	2.95	13.1
>40	8	5.04	13.1	2.91	12.5	5.01	15.6	2.90	12.8

Because of the rather good correlation between $t_{\frac{1}{2}}$ and L, BM_{th} and BM_s these relations were analysed in more detail according to the linear regression method.

References p. 682

D. Regression analysis and normal values in the galactose group

If, for the regression equation, $t_{\frac{1}{2}}/L = y$ at age x, it appears that:

$$\hat{y} = 7.63 + 0.0151\,x$$

with a correlation coefficient, $r = 0.235$. The standard error m_r of r is:

$$m_r = \frac{1-r^2}{\sqrt{n}} = 0.100$$

According to statistics a reliable correlation exists between x and y if $r > 3\,m_r$. In this analysis $r = 2.35 \times m_r$ is found, so that it is possible that $t_{\frac{1}{2}}/L$ is slightly related to age. If $t_{\frac{1}{2}}/BM_{th} = z$ at age x:

$$\hat{z} = 13.85 + 0.0017\,x$$

with a correlation coefficient, $r = 0.0153$. Standard error

$$m_r = 0.105;\ r = 0.146\,m_r$$

With $t_{\frac{1}{2}} \cdot BM_s = v$ at age x:

$$\hat{v} = 7.46 + 0.0175\,x \text{ with } r = 0.226$$
$$m_r = 0.100;\ r = 2.26\,m_r$$

From these analyses, it can be concluded that the age dependence of $t_{\frac{1}{2}}$ may be largely or entirely overcome by introducing $t_{\frac{1}{2}}/L$, $t_{\frac{1}{2}}/BM_{th}$ and $t_{\frac{1}{2}} \cdot BM_s$ in the galactose test.

As the correlation of $t_{\frac{1}{2}}$ with BM_s is probable from a physiological point of view, the most interesting effect, the relation between $t_{\frac{1}{2}} \cdot BM_s$ and age is given in Fig. 4.

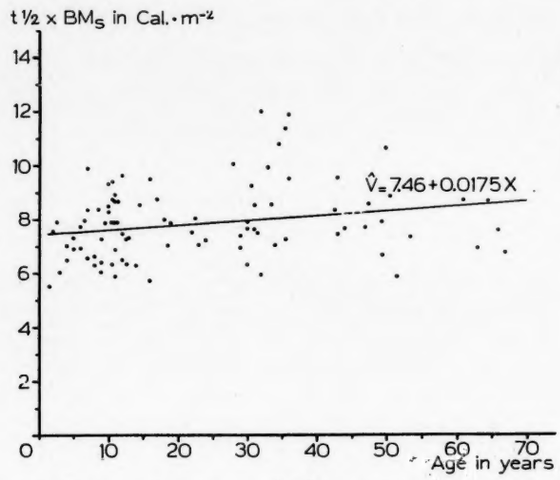

Fig. 4. Relation between age and $t_{\frac{1}{2}} \cdot BM_s$ in the control group of galactose.

The above-mentioned values may very suitably be used as normal standards in the galactose test. In Table III, their means and ranges are summarized, assuming a normal distribution.

TABLE III

"NORMAL" VALUES OF $t_{\frac{1}{2}}/L$, $t_{\frac{1}{2}}/BM_{th}$ AND $t_{\frac{1}{2}} \cdot BM_s$ FOR GALACTOSE WITH THEIR RANGES, BASED ON 90 INDIVIDUALS, ALL OLDER THAN ONE YEAR OF AGE

for σ and σ_m see [1].

	Mean	$\pm 2\sigma_m$ *range of the mean*	$\pm 2\sigma$ *range of observations*
$t_{\frac{1}{2}}/L$	7.98	7.75– 8.20	5.7 –10.3
$t_{\frac{1}{2}}/BM_{th}$	13.9	13.5 –14.3	10.0 –18.0
$t_{\frac{1}{2}} \cdot BM_s$	7.87	7.60– 8.14	5.30–10.5

References p. 682

It may be concluded from Fig. 1 and Table I that in adults $t_{\frac{1}{2}}$ is only slightly or not at all dependent on age. This explains why COLCHER and co-workers[14], who described good results with their "galactose-removal constant" in adults, did not observe the influence of age in the disappearance of galactose. We were able to calculate "normal" $t_{\frac{1}{2}}$-values from the investigations of COLCHER and co-workers[14]. From their control group, consisting of 6 normal adults and 4 patients with no clinical signs of liver disease, the mean value $t_{\frac{1}{2}} = 12.6$ could be calculated. Assuming, for his control group, the mean length (1.67 m), the mean BM_{th} (1.01 Cal. min^{-1}) and the mean BM_s (0.592 $Cal \cdot m^{-2} \cdot min^{-1}$) of the adults of our group, it appears that:

$$\frac{t_{\frac{1}{2}}}{"L"} = \frac{12.6}{1.67} = 7.6; \frac{t_{\frac{1}{2}}}{"BM_{th}"} = \frac{12.6}{1.01} = 12.5 \text{ and } t_{\frac{1}{2}} \cdot "BM_s" = 12.6 \cdot 0.592 = 7.46,$$

these mean values being on the lower limit of our mean results in 90 controls (Table III).

It is known[1, 15] that the liver plays a dominant part in the elimination of galactose. This activity may, in approximation, be expressed with the reaction constant K in equation (2) of [1]:

$$K = \frac{0.693}{t_{\frac{1}{2}}} \text{ min}^{-1}$$

If we assume that $t_{\frac{1}{2}} \cdot BM_s = 7.87$ (Table III) is constant with respect to age-dependence, it follows from the above equation that the activity of the liver may be expressed as:

$$K_{BM_s} = \frac{0.693 \cdot BM_s}{7.87} \text{ min}^{-1} = 0.088\, BM_s \text{ min}^{-1}$$

The above reaction constant K_{BM_s} of galactose (loading 0.5 g/kg b.w.) may then be seen as an age-independent mean standard of the potential activity of the liver in normal individuals.

It is evident from Fig. 4 that the elimination capacity of the liver (K) for galactose parallels BM_s. This may be caused by a relationship (circulatory, energetic), expecially as we found in two patients with myxedema decreased rates of elimination of galactose and of bromsulfalein. This agrees with the investigations of DONE *et al.* and BROWN *et al.* (see under *B*).

E. Regression analysis and normal values in the bromsulfalein group

In this group we found results analogous to those for galactose (under *D*).
If $t_{\frac{1}{2}}/L = y$ at age x:

$$\hat{y} = 2.63 + 0.0048\, x \text{ with } r = 0.168;\ m_r = 0.145;\ r = 1.16\, m_r$$

For $t_{\frac{1}{2}}/BM_{th} = z$ at age x:

$$\hat{z} = 4.68 + 0.0004\, x;\ r = 0.007;\ m_r = 0.149;\ r = 0.046\, m_r$$

With $t_{\frac{1}{2}} \cdot BM_s = v$ was found:

$$\hat{v} = 2.57 + 0.0077\, x;\ r = 0.258;\ m_r = 0.139;\ r = 1.86\, m_r$$

From these analyses it may be concluded that $t_{\frac{1}{2}}/L$, $t_{\frac{1}{2}}/BM_{th}$ and $t_{\frac{1}{2}} \cdot BM_s$ are not at all or only slightly dependent on age. Fig. 5 gives the results for $t_{\frac{1}{2}} \cdot BM_s$.

Table IV summarizes these relations as "normal" values in the bromsulfalein test, assuming a normal distribution.

References p. 682

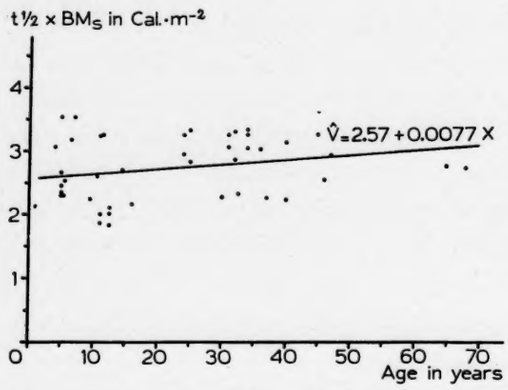

Fig. 5. Relation between age and $t_{\frac{1}{2}} \cdot BM_s$ in the control group of bromsulfalein.

TABLE IV

"NORMAL" VALUES OF $t_{\frac{1}{2}}/L$, $t_{\frac{1}{2}}/BM_{th}$ AND $t_{\frac{1}{2}} \cdot BM_s$ FOR BROMSULFALEIN WITH THEIR $\pm$ 2 σ RANGES, BASED ON 45 CONTROLS

	Mean	$\pm 2\sigma_m$ *range of the mean*	$\pm 2\sigma$ *range of observations*
$t_{\frac{1}{2}}/L$	2.74	2.59–2.89	1.78–3.70
$t_{\frac{1}{2}}/BM_{th}$	4.69	4.41–4.97	2.85–6.53
$t_{\frac{1}{2}} \cdot BM_s$	2.75	2.62–2.88	1.85–3.65

To compare our "normal" values with those of other investigators their results were converted to those of our terminology.

Some investigators in their studies with adults recorded sufficient data to calculate $t_{\frac{1}{2}}$ but they did not mention the subjects' age, sex, body length etc. For this reason we assumed for all their *adults* the mean values recorded under *D*. ($"L" = 1.67$; $"BM_{th}" = 1.01$ and $"BM_s" = 0.592$), and converted with the aid of these figures all results into the age-"independent" values: $t_{\frac{1}{2}}/"L"$, $t_{\frac{1}{2}}/"BM_{th}"$ and $t_{\frac{1}{2}} \times "BM_s"$.

Only the results described by DOST and co-workers[16] were obtained in children; their ages varied from 2 months to 12 years. These authors did not mention the children's body length, so this was approximately deduced from their body weight. As our age-limit was one year, only children older than this were included in our discussion.

Table V summarizes the converted, normal data obtained in children and adults.

TABLE V

MEAN "NORMAL" VALUES OF $t_{\frac{1}{2}}/"L"$, $t_{\frac{1}{2}}/"BM_{th}"$ AND $t_{\frac{1}{2}} \cdot "BM_s"$ OF BROMSULFALEIN FROM LITERATURE, COMPARED WITH OUR RESULTS

Authors	*Age group*	*Dose of bromsulfalein*	*Number*	*Means of:* $t_{\frac{1}{2}}/"L"$	$t_{\frac{1}{2}}/"BM_{th}"$	$t_{\frac{1}{2}} \cdot "BM_s"$
INGELFINGER *et al.*[17]	Adults	150 mg/sq. m.	158	2.94	4.85	2.90
LAVERS *et al.*[18]	Adults	5 mg/kg b.w.	42	2.22	3.67	2.19
GOODMAN[19]	Adults	5 mg/kg b.w.	6	2.70	4.45	2.66
BIRKENHÄGER[20]	Adults	4 mg/kg b.w.	13	2.04	3.37	2.01
DOST *et al.*[16]	Children	5 mg/kg b.w.	7	3.99	7.68	3.58
This investigation	Children and adults	5 mg/kg b.w.	45	2.74	4.69	2.75

References p. 682

From the control groups in Table V it is evident that the mean results of INGELFINGER and GOODMAN are in fair agreement with our own values. Those of LAVERS and BIRKENHÄGER, however, are significantly lower. The high values obtained by DOST *et al.* may be due to the fact that their control group cannot be considered as normal. It was composed of 7 children older than one year, of which 3 had heart disease and one myxedema. Both in the galactose and in the bromsulfalein test we found decreased elimination rates in patients with heart disease and with myxedema. This may be the reason why these authors did not notice a higher disappearance rate of bromsulfalein in their "normal" children than other investigators found in adults.

If we assume that the mean of $t_{\frac{1}{2}} \cdot BM_s$ is constant with respect to age-dependence, it follows (analogously as under *D*) that:

$$K'_{BM_s} = \frac{0.693 \cdot BM_s}{2.75} min^{-1} = 0.252\ BM_s\ min^{-1}$$

In this equation the specific reaction constant K'_{BM_s} may then be considered as a standard for the total capacity of the liver for bromsulfalein (loading 5 mg/kg b.w.) in normal individuals. As was found for galactose (under *D*), the constant of disappearance, K'_{BM_s} of bromsulfalein, parallels the standard basal metabolic rate (BM_s).

SUMMARY

1. The half-life ($t_{\frac{1}{2}}$) of galactose (0.5 g/kg b.w.) and of bromsulfalein (5 mg/kg) was used as a measure for the functional capacity of the liver in respectively 90 and 45 controls all over 1 year of age.
2. It was found both for the galactose and bromsulfalein group that in infants and children the elimination rates were considerably higher ($t_{\frac{1}{2}}$ lower) than in adults.
3. Although the explanation of this physiological age-dependence is complex, 5 factors were discussed which may be related to this phenomenon.
4. From a physiological and practical point of view we tried to eliminate this age-dependence.
   For that reason the relationships between age and $t_{\frac{1}{2}}$/bodyweight (BW), $t_{\frac{1}{2}}$/body length (L), $t_{\frac{1}{2}}$/surface area (SA), $t_{\frac{1}{2}}$/theor. bas. met. rate (BM_{th}), $t_{\frac{1}{2}} \times$ distribution volume (V) and $t_{\frac{1}{2}} \times$ standard bas. met. rate (BM_s) were studied, assuming a linear regression. These analyses showed that both for galactose and bromsulfalein, $t_{\frac{1}{2}}/L$, $t_{\frac{1}{2}}/BM_{th}$ and $t_{\frac{1}{2}} \cdot BM_s$ are slightly or not at all dependent on age, whereas $t_{\frac{1}{2}}/BW$, $t_{\frac{1}{2}}/SA$ and $t_{\frac{1}{2}} \cdot V$ appeared to be clearly related to age.
5. For this reason $t_{\frac{1}{2}}/L$, $t_{\frac{1}{2}}/BM_{th}$ and $t_{\frac{1}{2}} \cdot BM_s$ for galactose and bromsulfalein were introduced as age-"independent" standards, of which the means and normal ranges are given.
6. Assuming a first order disappearance of galactose and of bromsulfalein and an age-independence of *e.g.* $t_{\frac{1}{2}} \cdot BM_s$, the capacity of a normal liver to eliminate each of these substances may be combined into a simple equation. For galactose this is $K_{BM_s} = 0.088\ BM_s\ min^{-1}$; and for bromsulfalein, $K'_{BM_s} = 0.252\ BM_s\ min^{-1}$.
7. As galactose and bromsulfalein are chemically totally different, it is possible that the hepatic elimination of many substances is related to the basal metabolic rate.
8. It is possible that with increase of age, the decreasing metabolic rate, the decreasing relative liver mass and a decreasing relative hepatic blood flow play a part in the age-dependence of this kind of liver function test.

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PHYSIOLOGISCH- UND PATHOLOGISCH-CHEMISCHE UNTERSUCHUNGEN IN INDONESIEN

II. SERUM-UNTERSUCHUNGEN AN EUROPÄERN

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In einer früheren Veröffentlichung[1] berichteten wir über Arbeiten zur Ermittelung von Standard-Werten im Serum klinisch gesunder Indonesier.

Wir fanden dabei bei einigen Bestandteilen des Serums verglichen mit Normalwerten gesunder Menschen unter gemässigten Klimaten, in der europäischen und nordamerikanischen Literatur, beträchtliche Unterschiede.

Wie aus nachstehender Tabelle I zu ersehen ist, fanden wir die auffallendsten Unterschiede bei den Gesamteiweiss- und Lipoid-Phosphor-Werten. Minder beträchtliche Unterschiede zeigten sich bei den Albuminen, Globulinen, den Neutralfetten und Fettsäuren.

TABELLE I

	Einheit	*Geschlecht männl./weibl.*	*Mittelwerte indonesisch (gefunden)*	*Mittelwerte europäisch (aus Literat.)*
Gesamteiweiss	g%	m	8.13	7.20
		w	7.80	
Albumin	g%	m	5.40	5.20
		w	5.17	
	%	m	66.3	72.2
		w	66.2	
Globulin	g%	m	2.73	2.0
		w	2.64	
	%	m	33.7	27.8
		w	33.8	
Gesamtlipide	mg%	m	1093	909
		w	1084	
Lipoid-Phosphor	mg%	m	6.36	8.0
		w	6.65	
Gesamtphosphatide	mg%	m	149.5	188
		w	156.0	
Neutralfett und Fettsäuren	mg%	m	713.5	735
		w	703.0	589

Es interessierte uns nun, ob der Unterschied zwischen den Literaturangaben und unseren Analysenresultaten auf rassischen Arteigenschaften beruht, oder etwa

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Literatur S. 685

klimatisch bedingt ist. Wir bestimmten daher im Serum von 18 Europäern und Amerikanern (der weissen Rasse), die mindestens 6 Monate in Indonesien verbracht hatten, die oben angegebenen, abweichend befundenen Serumwerte. In Tabelle II sind die Resultate dieser Arbe verglichen mit den entsprechenden Werten der ersten Mitteilung.

TABELLE II

	Einheit	*Geschlecht m/w*	*Gesamt-abweichung*	*Indon.*	*Mittelwerte Eur./Am.*[1]	*Europäer*[2]
Gesamteiweiss	g%	m	7.80–8.50	8.13	8.16	7.20
		w	7.80–8.37	7.80	8.07	
Albumin	g%	m	5.19–6.06	5.40	5.56	5.40
		w	5.41–5.88	5.17	5.68	
	%[3]	m		66.3	68.5	72.2
		w		66.2	70.4	
Globulin	g%	m	1.85–2.93	2.73	2.61	2.0
		w	2.17–2.65	2.64	2.39	
	%[3]	m		33.7	31.5	27.8
		w		33.8	29.6	
Gesamtlipide	mg%	m	900–1300	1093	1126	909
		w	900–1220	1084	1052	
Lipoid-Phosphor	mg%	m	6.2–8.8	6.36	7.55	8.0
		w	6.5–9.8	6.65	7.75	
Gesamt-Phosphatide	mg%	m	146–209	149.5	175.5	188.0
		w	152–230	156.0	182.0	
Neutralfett u.		m	507–877	714	703	735
Fettsäuren		w	540–818	703	643	589

[1] Europäer (und Amerikaner) in Indonesien.
[2] Europäer nach europäischen Literaturangaben.
[3] Anteil am Gesamteiweiss.

Die Gesamteiweiss-Werte liegen in beiden untersuchten Gruppen gleich hoch. Die Werte der Albumin/Globulin Fraktion und die des Lipoid-Phosphors und damit auch die der Phosphatide liegen zwischen den indonesischen und den europäischen Werten. Jedenfalls sind auch diese durch das Klima beeinflusst. Der Gehalt an Gesamtlipide liegt bei Indonesiern eher noch etwas höher, was wohl mit der allgemein gültigen Ansicht, dass die hier lebenden Europäer fettreicher essen als die Indonesier, zu erklären sein dürfte. Diese Werte sind höher als die europäischen Literaturangaben. Die Neutralfette und Fettsäuren sind bei allen drei Gruppen ungefähr gleich. Auffallend, aber nicht überraschend, ist die geringe Streuung der Untersuchungsergebnisse bei den in Indonesien lebenden Europäern. Die Erklärung hiefür dürfte wohl sein, dass diese Gruppe im Grunde genommen, trotz verschiedenem Körperbau, viel einheitlicher zu sein scheint als die untersuchten Indonesier. Hierauf wurde bereits in der ersten Mitteilung hingewiesen.

Es sei noch erwähnt, dass der grosse Arzt und Physiker ROBERT MEYER, anlässlich seiner Untersuchungen im Hafen von Soerabaya (Java) im Jahre 1840 erstmalig auf Unterschiede in der Blutfarbe der hier untersuchten Matrosen und der bei europäischen Untersuchungen beobachteten Blutfarbe hinwies. Diese Beobachtungen regten seine Gedanken über die Energie-Gesetze an, die er erstmals in *Liebigs Annalen* im Jahre 1842 veröffentlichte[2].

Literatur S. 685

ZUSAMMENFASSUNG

In einer früher veröffentlichten Mitteilung über die Aufstellung von Normalwerten im Serum von Indonesiern wurden Unterschiede bei dem Gesamteiweiss-, Gesamtlipide- und Phosphatide-Gehalt gegenüber europäischen und amerikanischen Literaturangaben gefunden. In dieser Veröffentlichung wird berichtet, dass Untersuchungen an seit mehreren Monaten in Indonesien verweilenden Europäern und Amerikanern zum Teil dieselben Wert, zum Teil Mittelwerte zwischen den europäischen Literaturangaben und den indonesischen Analysen-Werten ergaben. Die Unterschiede sind somit als klimatisch, nicht jedoch als rassisch bedingt aufzufassen.

SUMMARY

ANALYSES OF NORMAL AND PATHOLOGIC SERA IN INDONESIA

II. SERA OF EUROPEANS

A previous communication reported standard values of components found in Indonesian sera; differences between the total protein, total lipid and phosphatide content of Indonesian sera as compared with the values found in the European and American literature were discussed. This paper reports on analyses carried out on sera of Europeans and American whites who had been residing in Indonesia for several months. The results obtained were partly the same as those found in Indonesian sera, and partly they lay between the latter and the values reported in European literature. This indicates that the differences observed were climatic and not racial.

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ROUTINE MICROESTIMATION OF IRON IN HEMOGLOBIN

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INTRODUCTION

Accurate and easily performed hemoglobin measurement is increasingly required. A "plus-minus one gram" report is too inaccurate, and the extensive use of iron therapeutically has made it essential to follow up changes of as little as 0.3–0.5 g% in the hemoglobin level. Much work has already been done in order to find suitable standards[1,2] for hemoglobin analysis and to develop suitable methods[3,4]. SUNDERMAN[4] stresses the desirability of the use of iron as a reference standard because of its stability, reliability and ready availability. He has also shown that iron estimation is within the bounds of possibility of most clinical laboratories[5]. We have attempted to work out a method for iron determination which is not only suitable for hemoglobin standardization, but is so simple and reliable that it can compete with any of the usual biochemical tests and therefore can be used as a routine test for the exact measurement of hemoglobin in a large number of specimens.

MATERIALS AND METHODS

The principle of the method is similar to that of THOMPSON[6] and WONG[7]; and the reaction between iron and thiocyanate. Blood is treated with concentrated sulfuric acid and persulfate, then the reagent, a thiocyanate–isobutyl alcohol solution, is added, and after mixing and clarifying the colour intensity is measured.

REAGENTS

1. Sulfuric acid, conc. C.P.
2. Potassium persulfate (saturated solution): 8 g of a good grade reagent shaken with 100 ml of distilled water.
3. Colour reagent: dissolve 30 g of potassium thiocyanate in 100 ml of distilled water, and add 4 ml of acetone and 500 ml of isobutyl alcohol.
4. Iron standard solution: dissolve 0.8635 g of $FeNH_4(SO_4)_2 \cdot 12H_2O$ in the minimum amount of distilled water, add 2 ml of concentrated sulfuric acid, let cool and dilute to 1000 ml with distilled water. One ml of this standard solution contains 100 μg of ferric iron; 0.1 ml, the amount used, corresponds to 50 mg % of iron under the conditions. If obtainable, a certified iron standard solution or one prepared from pure iron wire, containing the same amount of iron as given above should be used.

PROCEDURE

In a centrifuge tube of about 15-ml capacity, 5 drops of distilled water were placed. Exactly 0.02 ml of blood, followed by 0.3 ml of sulfuric acid and 0.5 ml of potassium persulfate solution were added. After cooling, 5 ml of colour reagent were added and the mixture was immediately shaken vigorously and centrifuged at high

References p. 690

speed for 10 min. Enough of the clear, coloured supernatant solution was removed for colorimetry with a dry pipette*. Readings were taken on the Klett** colorimeter. (Any similar instrument is suitable.) For every batch of tests one blank containing the reagents only, and one standard using 0.1 ml of iron standard solution (equivalent to 10 μg of ferric iron) were carried out simultaneously. The zero setting of the colorimeter was made against isobutyl alcohol.

CALCULATION

$$\frac{50}{(S-B)} \cdot (U-B) = \text{mg \% of iron in the sample.}$$

The hemoglobin can be directly calculated by the following formula:

$$\frac{14.7}{(S-B)} \cdot (U-B) = \text{g \% of Hb.}$$

since

$$\frac{\text{mg of iron per 100 ml}}{3.40} = \text{g of Hb per 100 m,}$$

where S = reading of standard, B = reading of blank, U = reading of unknown.

DISCUSSION

We have repeated the check on hemoglobinometry which was done in the U.S.A.[4] by requesting various laboratories to measure the hemoglobin contents of two blood samples. The purpose of comparison was to produce a value for precision and to reduce technical errors. However, the range of variation was very great (Table I). Most of the

TABLE I

HEMOGLOBIN VALUES OBTAINED ON ANALYSIS OF THE SAME TWO SPECIMENS BY DIFFERENT LABORATORIES USING THEIR ROUTINE METHODS

Laboratory	*Method*	*Hb value (g %)*		*Deviation from real value (g %)*	
		Specim. 1	*Specim. 2*	*Specim. 1*	*Specim. 2*
A	Acid hematine	11.4	8.1	—0.2	—1.2
B	—	10.2	7.8	—1.4	—1.5
C	—	13.1	9.7	+1.5	+0.4
D	—	11.9	8.6	+0.3	—0.7
E	—	15.7	10.6	+4.1	+4.1
F	—	10.8	9.6	—0.8	+0.3
G	Carboxy Hb	12.4	10.3	+0.8	+1.0
H	—	14.1	11.2	+2.5	+1.9
I	—	13.1	9.9	+1.5	+0.3
J	Cyanomet-Hb	11.5	9.8	—0.1	+0.5
K	—	11.7	9.9	+0.1	+0.6
L	—	12.2	10.4	+0.6	+1.1
M	—	13.6	12.3	+2.0	+3.0
N	—	10.1	10.0	—1.5	+1.7
O	—	15.0	13.0	+3.4	+3.7
P	—	13.1	9.3	+1.5	0
S	—	11.4	9.8	—0.2	+0.5

* To improve speed of work a rubber bulb for pipetting is recommended.

** A Klett-Summerson photoelectric colorimeter was used in the present work.

References p. 690

laboratories used the cyanomethemoglobin[3] method but only two had the standards prepared by DRABKIN. Some others had used them previously but had abandoned them, and one worker found them unsatisfactory owing to too high values. We concluded from the survey that there is still need for a new method of high reliability in hemoglobinometry.

TABLE II

COMPARISON OF HB VALUES AND TIME OF THE TEST BY THREE DIFFERENT METHODS

No. of sample	*Hemoglobin (g %)*		
	WONG's[7] *method*	DRABKIN's[3] *method*	*Present method*
1	12.4	11.4	12.0
2	15.3	16.1	15.1
3	14.4	14.7	13.7
4	16.3	19.0	16.3
5	17.8	18.5	18.0
6	14.7	14.3	14.7
7	10.2	9.4	10.0
8	16.1	15.5	15.8
9	16.1	16.6	15.9
10	16.5	16.3	16.3
11	12.0	13.6	12.4
12	14.3	14.1	14.3
13	16.4	17.4	16.8
14	8.1	9.2	7.8
15	15.3	16.4	15.7
16	18.2	20.1	18.1
17	5.4	6.3	6.0
18	12.2	11.4	11.8
19	11.3	10.7	11.1
20	16.4	16.3	16.1
21	14.6	14.4	14.4
22	18.1	16.5	17.8
23	10.3	11.6	10.3
24	5.5	6.0	5.7
25	15.0	14.7	15.1
26	14.1	13.0	14.4
27	8.1	9.8	8.4
28	17.7	18.4	17.5
29	9.6	10.4	9.8
30	15.1	17.2	15.6
Time of performance	3 h 36 min	44 min	81 min

The method proposed is simple enough for routine work and its results compare favourably with WONG's[7] (Table II). The linearity of light absorption is very good, and Beer's law is obeyed over a wide range of concentration (Fig. 1).

WONG's[7] method is most often used for hemoglobin standardization. It is not suitable for routine hemoglobinometry because it lasts too long, (see Table II), requires too much blood, and the colour produced in the reaction fades; therefore, the greater

References p. 690

the number of tests, the less reliable the results. The test proposed takes little time if performed by experienced technicians (Table II). It requires the same amount of blood as the other routine hemoglobin methods (*e.g.* 0.02 ml) and the colour is stable for a long time in all the necessary concentrations (Table III).

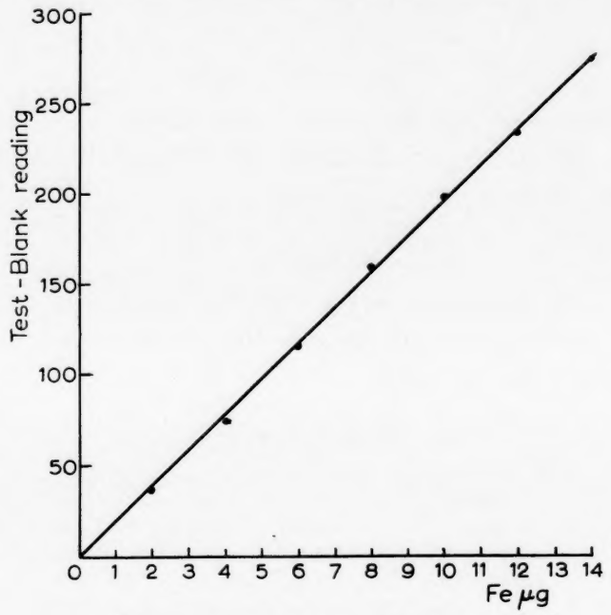

Fig. 1. Curve of iron standards.

As far as possible, good grades of analytical reagents should be used, but glass-distilled water is unnecessary, since all iron contaminations are deducted with the blank readings. Deproteinization is omitted and with it the main source of error of WONG's method[7]; sodium tungstate is not used.

TABLE III

STABILITY OF COLOUR OF THE IRON–THIOCYANATE COMPLEX

Hb concentration (*g %*)	*Colorimeter reading*				
	5 min	*10 min*	*30 min*	*60 min*	*120 min*
3	87	90	90	91	93
6	121	124	124	124	125
9	170	172	173	173	173
12	208	212	212	213	216
15	244	251	251	253	253
18	282	285	286	290	291
21	325	328	328	330	329
Blank 0	50	51	51	52	52

However, a few precautions must be taken in order to ensure precise results.

a) The blood and the colour reagent must be exactly measured.
b) Clean glassware must be used, and any contact with iron avoided to prevent further reaction of the coloured solution. The tube must be shaken well immediately after addition of the colour reagent, otherwise the colour fades.
c) The colorimeter tube and the pipette used must be free from water to avoid emulsification with the isobutyl alcohol and consequently faulty light absorption.

References p. 690

However, if emulsification occurs, the sample might be saved by the addition of one drop of propyleneglycol which clarifies the solution.

With the observance of these precautions and with average skill, this method could be very useful in routine hemoglobinometry, whenever it is possible to exchange working time for greater precision.

ACKNOWLEDGEMENTS

Our thanks are due to Dr. H. MENACHEM for calling our attention to the problem, Prof. F. RAPPAPORT for his help and many useful suggestions, and Dr. MUNDEL for help in preparing the manuscript.

SUMMARY

A new method for hemoglobinometry based on micro iron estimation is presented. It is reliable, easily performed, and suitable for routine examinations, as well as for the standardization of other methods.

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ESTEROLYSIS OF HUMAN SERUM AFTER INTRAVENOUS INFUSION OF HEPARIN*

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The biological shape of the curve of postheparin esterase in human serum is influenced by several factors. We have studied to what extent the rapid decrease of postheparin esterase activity of human serum after a single dose of 5,000 u. of heparin reflects the increasing deficiency of the administered heparin, and to what extent this decrease can be prevented by modifying the usual method of administration and using a continuous drop infusion.

METHODS

The present work was based on our previous investigations on the heparin activation of serum esterases assessed by means of the substrates Tween 20, 60, 80[1–3]; the amount of liberated fatty acids (lauric, stearic, oleic) were estimated nephelometrically in a veronal buffer pH 7.9 in the presence of calcium ions. The investigations were carried out on a group of volunteers and patients, aged 18–56 years who were admitted to our clinical department for various digestive disturbances. Dosages were made as follows: half an hour after the administration of the basic dose of 5,000 u., an infusion of a further 10,000 u. dissolved in 300 ml physiological saline was administered in gradually increasing amounts. By the end of the first hour 500 u. had been infused, during the second hour 1,000 u., during the third hour 2,500 u. and during the 4th and 5th hours when infusion is completed 3,000 u. The blood specimens were taken on fasting, after an interval of 15 min and subsequently after 1, 2, 3, 4, and 5 h. The changes recorded during the comprehensive investigation of the metabolic effect of heparin by examining bound and non-esterified fatty acids, the blood sugar level and ketone bodies, changes of the dissociation curve and glycolysis in the erythrocytes, are summarized elsewhere.

RESULTS

Fig. 1 records the serum esterolysis activity in the substrate Tween 60. There are considerable individual variations in the level of the maximum activity immediately after the administration of heparin as well as in the subsequent course of the curves. After a single dose, the usual, more or less rapid decrease of activity, which is at a minimum during the 4th and 5th hour was recorded, but the postheparin esterase after an infusion had a quite different character. The maximum effect persists throughout the experiment on a high level except for a slight decrease at the end of the first hour; this is probably due to the relatively small dose of heparin administered in infusion form during this interval. Fig. 2 compares average values of postheparin esterase in human serum on different substrates after a single dose and infusion respectively. Table I summarizes the average values and standard deviations.

* Presented as a short communication at the Conference of the Institute of Human Nutrition, Prague, June 1958.

References p. 693

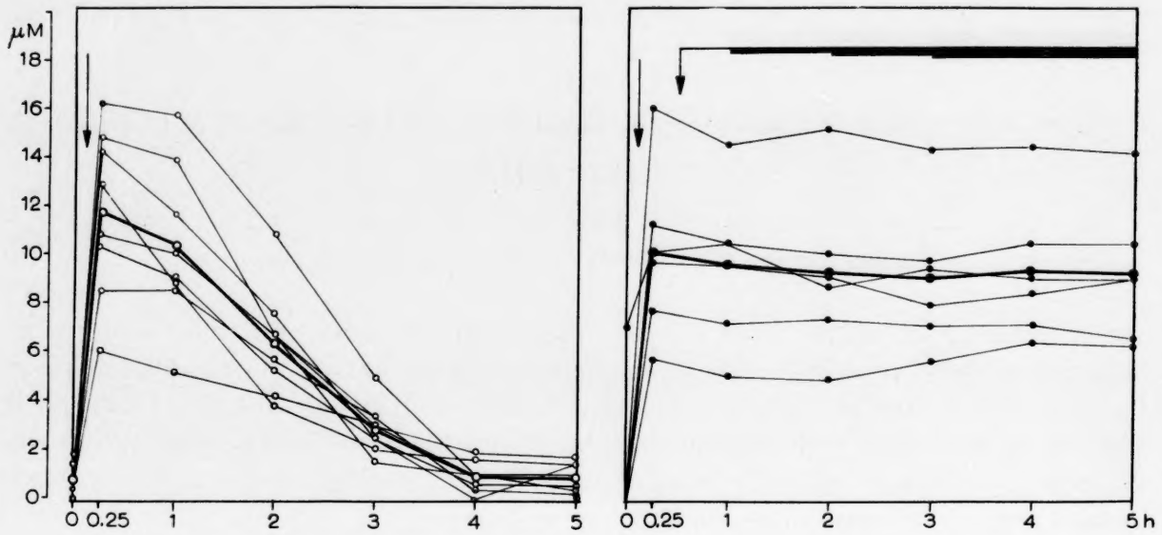

Fig. 1. Postheparin esterase activity in human serum (Tween 60) after 5.000 u. heparin (↓) and after heparin infusion (⎓) in increasing doses (500, 1,000, 2,500, 3,000, 3,000).

TABLE I

Hour	*Heparin 5,000 u.*			*Heparin infusion*		
	Tween 20	*Tween 60*	*Tween 80*	*Tween 20*	*Tween 60*	*Tween 80*
0.00	5.34 ± 0.94	0.71 ± 0.22	0.30 ± 0.84	4.47 ± 1.82	1.17 ± 2.85	0.0 ± 0.00
0.25	12.65 ± 2.37	11.70 ± 3.43	6.82 ± 3.63	11.67 ± 3.19	10.07 ± 3.49	6.47 ± 3.50
1.00	11.32 ± 1.96	10.31 ± 3.35	4.26 ± 3.79	10.85 ± 3.16	9.48 ± 3.25	5.95 ± 3.19
2.00	8.75 ± 1.26	6.30 ± 2.23	0.80 ± 1.19	10.33 ± 3.47	9.13 ± 3.43	5.18 ± 2.89
3.00	6.80 ± 0.99	2.81 ± 1.03	0.30 ± 0.84	10.98 ± 2.66	9.00 ± 3.00	5.27 ± 3.34
4.00	5.76 ± 1.19	0.94 ± 0.61	0.30 ± 0.84	11.10 ± 2.54	9.28 ± 2.88	4.80 ± 3.17
5.00	5.39 ± 1.11	0.90 ± 0.51	0.30 ± 0.84	10.90 ± 2.40	9.20 ± 2.89	5.00 ± 2.40

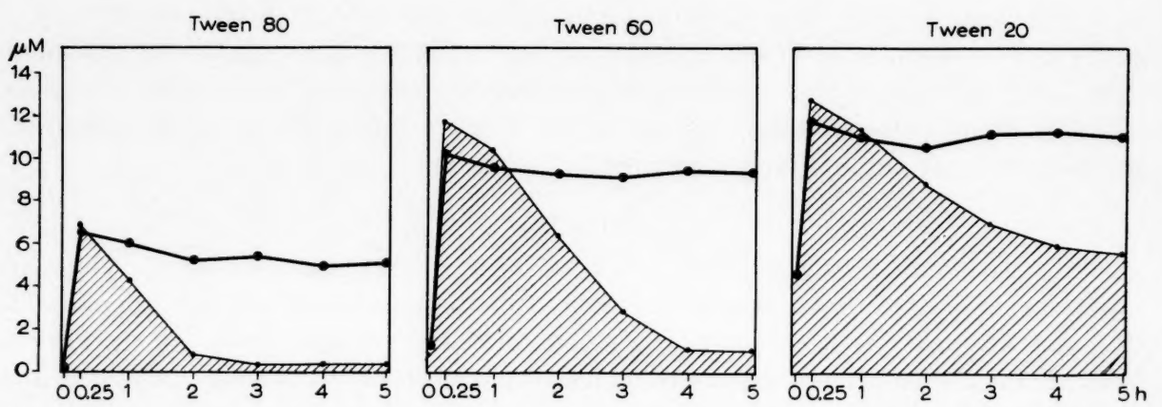

Fig. 2. Comparison of average activities of postheparin esterases in human serum after a single dose of 5,000 u. of heparin (line demarkating hatched area) and after heparin infusion (-●-●-●-).

DISCUSSION

This investigation was suggested by the work of JAQUES *et al.*[4], concerning the metabolism and excretion of heparin and its metabolites in urine. JAQUES showed that the excretion of the maximal metachromatic activity occurs during the first 40 min. Further valuable data which give a more precise idea of the changes undergone by intravenously administered heparin, are given in the work of EIBER AND

References p. 693

DANISHEFSKY[5-7], who investigated the clearance of heparin ^{35}S from the blood stream in dogs. These investigations suggest a certain parallelism between the decrease of intravascular postheparin esterase activity and the clearance of heparin from the blood stream, and possibly also its metabolism in the organism and the urinary excretion. At the same time a more accurate interpretation is possible of the mechanism of the decreasing postheparin esterase activity; this can be explained as exhaustion of the material necessary for the formation of this active enzyme by stressing the role of the increasing deficiency of the exogenous component.

We felt that these experiments should be reported because this method permits, as we have shown elsewhere[8], the investigation of the metabolic effects of heparin which cannot be produced in individuals with metabolism by means of a single dose; moreover it may help to elucidate the heparin activation of serum esterases.

SUMMARY

A method of administration of heparin in the form of a protracted drop infusion (15,000 u./5 h) is described which allows the investigation of the metabolic and enzymic effects of heparin in clinical experiments. The postheparin activity of serum esterases estimated by means of substrates Tween 20, 60 and 80 was maintained throughout the experiment on a high level.

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ORIGINE PLASMOCYTAIRE D'UNE GLOBULINE MYÉLOMATEUSE β_2

ÉTUDE DE SES RAPPORTS IMMUNOÉLECTROPHORÉTIQUES AVEC LA β_{2A}-GLOBULINE

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L'immunoélectrophorèse a été utilisée dans l'étude du sérum de myélome par GRABAR *et coll.*[1]. Ces auteurs ont décrit des modifications portant uniquement sur 2 types de globulines: les γ-globulines et la β_{2A}-globuline. Cette β_{2A}-globuline fut récemment isolée du sérum humain normal par HEREMANS, HEREMANS ET SCHULTZE[2]: c'est une glycoprotéine ayant les propriétés d'une pseudoglobuline, et possédant quelques rapports antigéniques avec les γ-globulines. Dans le myélome, cette globuline peut être considérablement augmentée ou au contraire disparaître totalement du diagramme.

Nous nous sommes attachés, dans un cas de myélome β_2 (Fig. 1A), à démontrer l'origine plasmocytaire de la globuline anormale et nous avons abordé l'étude de ses rapports antigéniques avec la β_{2A}-globuline.

ORIGINE PLASMOCYTAIRE DE LA GLOBULINE ANORMALE

L'origine plasmocytaire des globulines anormales décelées par l'électrophorèse des sérums de myélome est suggérée par de nombreux arguments cliniques et thérapeutiques. Cependant les arguments tirés d'analogies entre les vitesses électrophorétiques[3] des globulines sériques et tissulaires sont insuffisants. Les études immunochimiques sont délicates, étant donné les rapports antigéniques étroits existant entre les globulines anormales et les γ-globulines normales[4]; un essai d'identification immunologique a été récemment décrit par SERRE ET JAFFIOL[5]. Nous nous proposons tout d'abord de préciser l'origine de la globuline anormale dans un cas de myélome β_2 qui nous a été soumis récemment.

1. *Diagrammes électrophorétiques*

L'électrophorèse sur papier du sérum de malade met en évidence un sommet anormal, bien homogène, coloré intensément par la réaction à l'acide periodique /réactif de Schiff, qui possède une vitesse électrophorétique de β_2-globuline normale. Dans l'urine, on ne trouve pas de globuline anormale par électrophorèse sur papier ni par immunoélectrophorèse.

L'immunoélectrophorèse sur gélose tamponnée à pH 8.2 réalisée avec un sérum équin antisérum-humain-normal met en évidence un arc de précipitation β_2, très intense et curviligne. Il n'existe pas d'arc de précipitation au niveau de la β_{2M}-globuline (macroglobuline β_2); l'arc des γ-globulines normales n'est pas modifié; on ne

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Bibliographie p. 700

distingue plus l'arc β_{2A} habituel (Figs. 1A et 1B). Ce diagramme est identique à celui qu'ont décrit GRABAR *et coll.*[1] dans deux cas de myélome β_2.

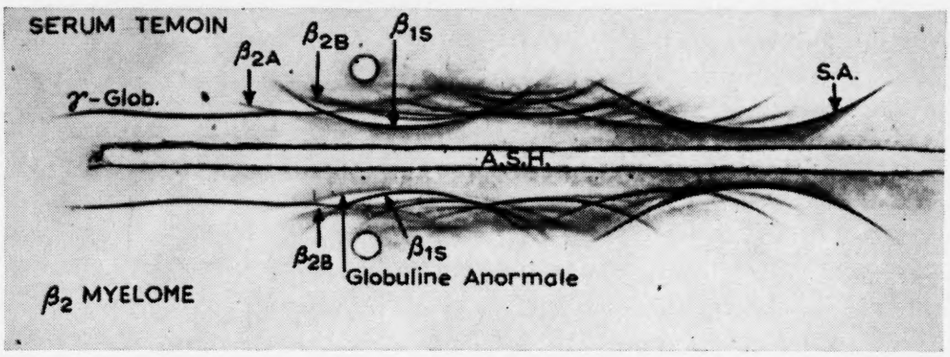

Fig. 1A. Étude immunoélectrophorétique d'un sérum d'adulte sain et du sérum de myélome β_2 réalisée avec le sérum équin antisérum-humain (A.S.H.).

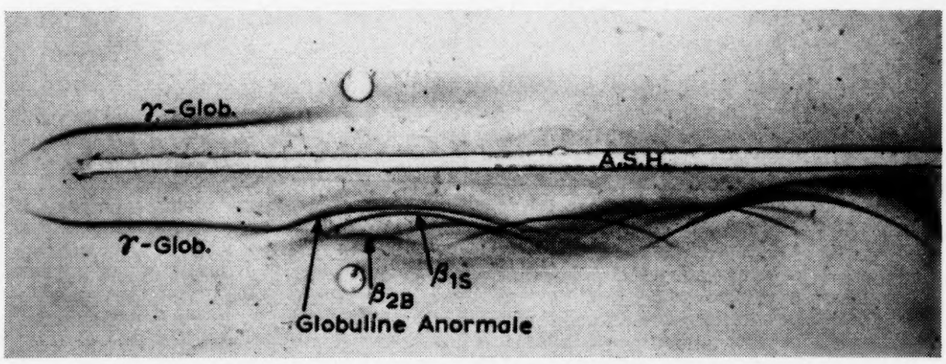

Fig. 1B. Étude avec ce même sérum d'une préparation de γ-globulines et du sérum du malade dilué au demi.

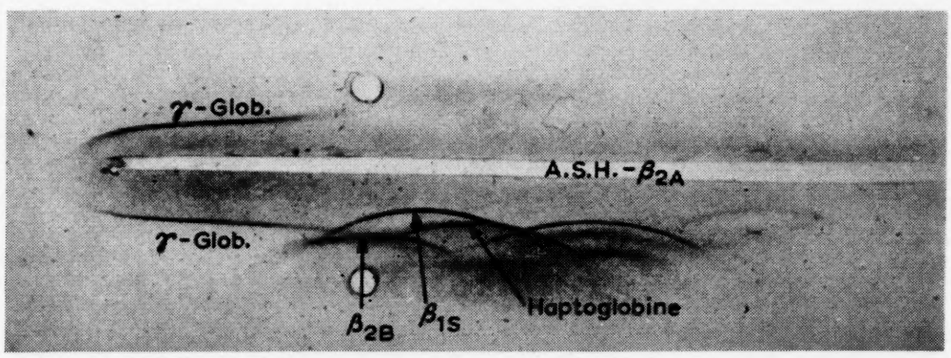

Fig. 1C. Mêmes préparations que dans la Fig. 1B étudiées avec le sérum antisérum-humain épuisé par un colostrum humain. L'arc des γ-globulines persiste, la β_{2A}-globuline anormale n'est plus révélée (comparer à la Fig. 1B).

2. *Caractérisation antigénique de la globuline anormale*

La globuline anormale possède un ou plusieurs déterminants antigéniques communs avec une globuline sérique normale, puisqu'elle se révèle avec l'immunsérum antisérum-humain-normal. Pour caractériser plus nettement cette globuline β_2 nous avons épuisé l'immunsérum par un adialysable de colostrum humain*.

* La totalité des globulines immunes de ce colostrum humain est représentée par une globuline identique à la β_{2A}-globuline normale (résultats non publiés).

Bibliographie p. 700

Un sérum humain normal, étudié avec cet immunsérum épuisé par le colostrum, donne un diagramme caractérisé dans la zone des β_2 et des γ-globulines par:
– la persistance des arcs de précipitation des γ-globulines et de la β_2-macroglobuline;
– la disparition totale de l'arc de la β_{2A}-globuline.

Ce même antisérum ne révèle pas l'arc β_{2A} particulier observé sur le diagramme du malade (Fig. 1C).

3. *Étude immunoélectrophorétique de la moelle osseuse*

Une petite quantité de moelle osseuse prélevée chez le malade est soumise à des lavages successifs par du sérum physiologique (voir SERRE ET JAFFIOL)[5]. Les différentes eaux de lavage sont concentrées par lyophilisation et étudiées en immunoélectrophorèse. Elles contiennent principalement de la sérumalbumine et un arc de précipitation β_2 dans les 6 premières eaux de lavages (Fig. 2A). Lorsque les eaux de lavage ne contiennent plus de protéines, on étudie directement la moelle osseuse en électrophorèse sur gélose. La révélation immunologique est réalisée avec: (a) un sérum équin antisérum-humain-normal (A.S.H.); (b) ce même immunsérum épuisé par le colostrum humain (A.S.H.-β_{2A}).

Sur les diagrammes, on retrouve un arc bien net, curviligne, de β_2-globuline, révélé par l'immunsérum A.S.H., mais non révélé par l'antisérum épuisé par le colostrum (Figs. 2A et 2B).

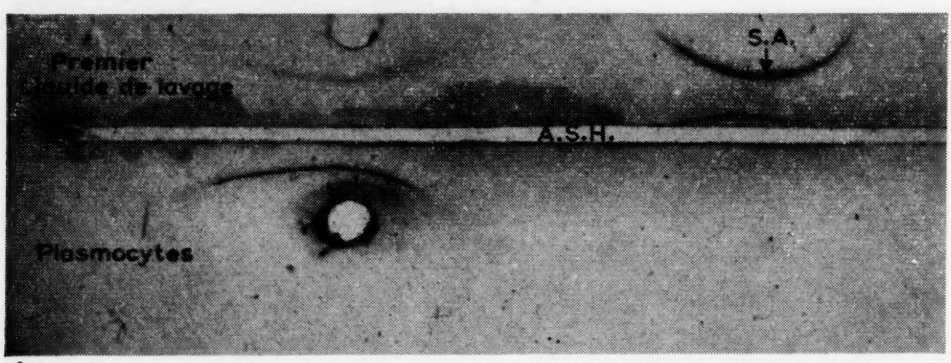

Fig. 2A. Étude des premiers liquides de lavage du fragment médullaire par du sérum physiologique (diagramme supérieur). L'arc de sérumalbumine est nettement prédominant. Les plasmocytes anormaux lavés ne contiennent qu'une seule fraction antigénique à vitesse de β_2-globuline.

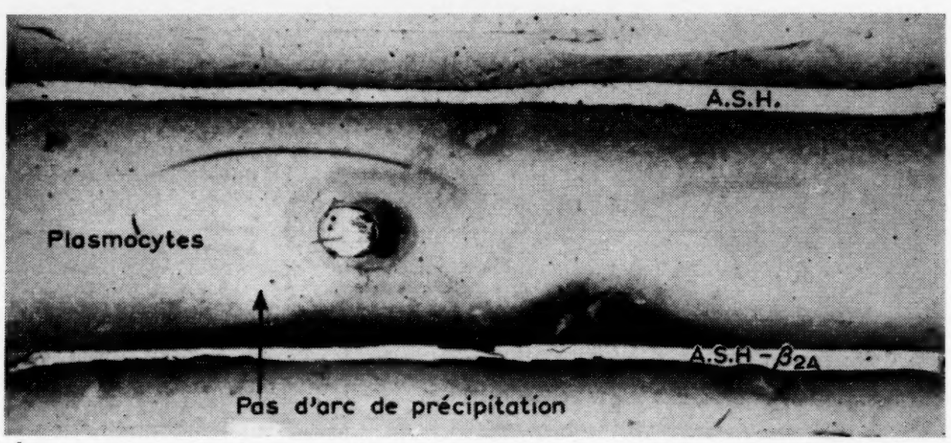

Fig. 2B. Étude de la purée plasmocytaire à l'aide des deux anti-sérums utilisés dans les Figs. 1B et 1C. Le sérum antisérum-humain épuisé par le colostrum (A.S.H.-β_{2A}) ne révèle aucune fraction dans cette préparation.

Bibliographie p. 700

4. *Discussion des résultats*

L'origine plasmocytaire des γ-globulines normales paraît actuellement très vraisemblable. Il nous suffira de rappeler que le mélange de suspensions cellulaires d'animaux immunisés avec des antigènes bactériens et de l'antigène homologue donne lieu à des phénomènes d'agglutination des bactéries sur les plasmocytes[6]. D'autre part, les anticorps marqués par la fluorescéine se localisent dans le cytoplasme et dans les noyaux des plasmocytes immatures[7,8].

D'autres arguments cliniques (chez des enfants atteints d'agammaglobulinémie congénitale) ou expérimentaux permettent d'attribuer aux plasmocytes normaux un rôle dans la synthèse des γ-globulines normales[9,10].

Il est donc nécessaire, pour étudier la globuline anormale d'un myélome, de rechercher un critère immunochimique de différenciation entre les γ-globulines normales et la globuline myélomateuse. Ce moyen nous a été fourni par l'utilisation simultanée, au cours de la révélation, d'un sérum antisérum-humain-normal et de ce même immunsérum épuisé par les protéines du colostrum humain. Un motif antigénique commun peut ainsi être mis nettement en évidence entre la β_{2A}-globuline normale et la globuline anormale de notre myélome β_2. Il existe d'autre part un motif antigénique commun entre la globuline β_2 décelée dans les plasmocytes anormaux et la globuline anormale du myélome étudié. Or, la β_{2A}-globuline normale étant disparue du diagramme sérique du malade, il est évident que c'est bien la globuline anormale qui tire son origine des cellules médullaires anormales. Un argument définitif est d'ailleurs apporté par l'étude immunoélectrophorétique du mélange de la purée plasmocytaire et du sérum du malade: en effet, cette préparation étudiée avec le sérum antisérum-humain montre l'existence d'un seul arc de précipitation possédant la vitesse électrophorétique de la globuline anormale.

RAPPORTS ANTIGÉNIQUES ENTRE LA GLOBULINE ANORMALE ET LA GLOBULINE β_{2A}

Nous avons essayé ensuite de préciser les rapports immunoélectrophorétiques entre la β_{2A}-globuline immune normale et la β_2-globuline anormale de notre myélome. Un sérum normal est mélangé à la purée plasmocytaire et étudié en immunoélectrophorèse (Fig. 2C). On observe deux arcs de précipitation correspondant à deux globulines β_2 de vitesses électrophorétiques différentes, mais qui se raccordent totalement au niveau de l'arc des γ-globulines. Ces deux arcs de précipitation possèdent

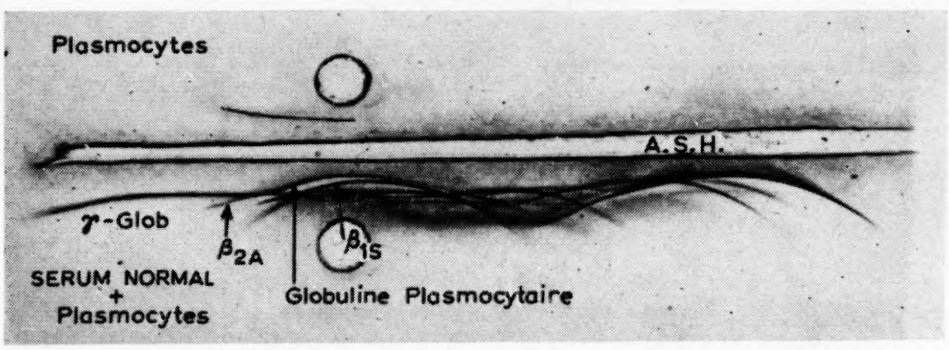

Fig. 2C. Un sérum normal enrichi en purée plasmocytaire, mis en présence de l'antisérum humain, montre un arc anormal se raccordant à l'arc β_{2A}-globuline normale.

Bibliographie p. 700

des concavités distinctes, qui traduisent des différences de vitesse de diffusion dans la gélose ou des différences de concentration. Inversement, l'enrichissement du sérum de myélome en β_{2A}-globuline normale confirme exactement les résultats précédents (Fig. 3A):

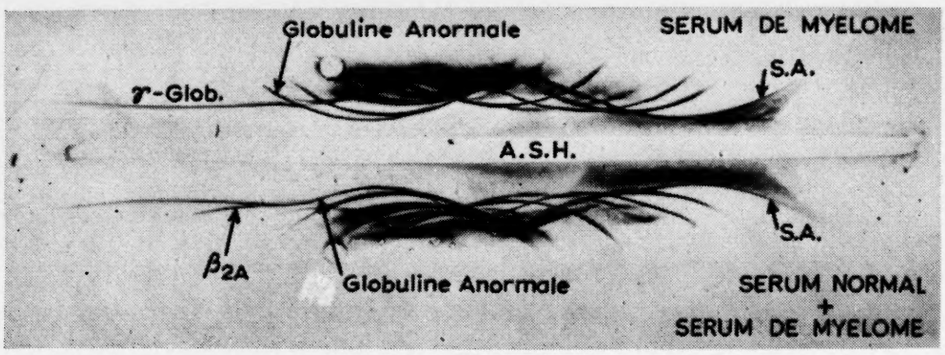

Fig. 3A. Étude du sérum de myélome (diagramme supérieur) et de ce même sérum enrichi en β_{2A}-globuline, réalisée avec le sérum équin antisérum-humain.

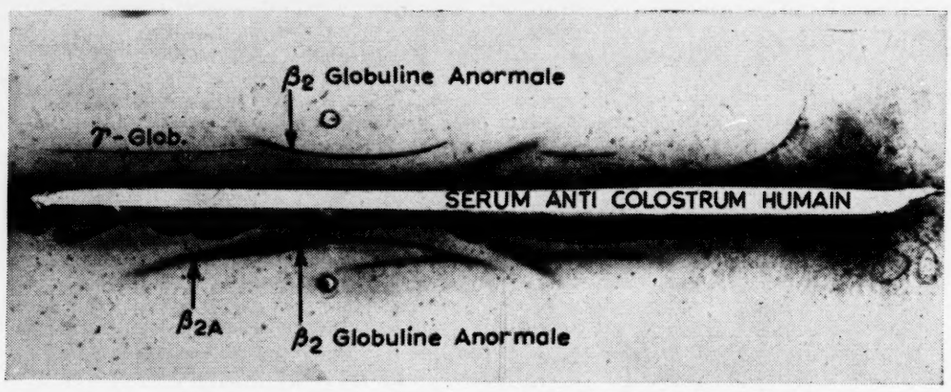

Fig. 3B. Ces mêmes préparations sont étudiées avec un sérum anti-colostrum humain révélant faiblement l'arc des γ-globulines et la globuline anormale, plus intensément la β_{2A}-globuline ajoutée au sérum de myélome. Cette globuline se raccorde totalement à l'arc de précipitation de la globuline anormale.

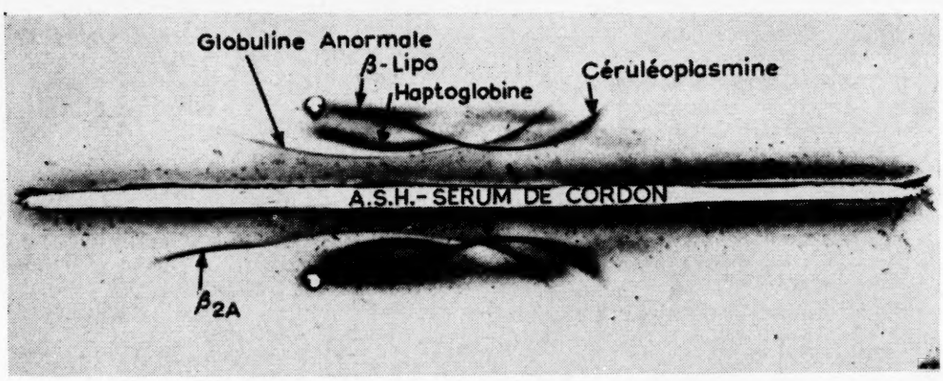

Fig. 3C. Ces mêmes préparations sont étudiées enfin à l'aide du sérum équin antisérum-humain épuisé par le sérum du sang de cordon ombilical, qui ne possède que les anticorps spécifiques de la β_{2A}-globuline, de la β_{2B}-globuline, des haptoglobulines et de la céruléoplasmine. On observe encore une identité antigénique totale entre la β_{2A}-globuline et la globuline anormale (diagramme inférieur).

Bibliographie p. 700

– différence de vitesse électrophorétique entre la β_{2A}-globuline normale et la globuline myélomateuse;

– différence de vitesse de diffusion dans la gélose de ces deux globulines;

– raccordement des deux arcs de précipitation au niveau de l'arc des γ-globulines, qui épouse à ce niveau la courbure de l'arc de la β_2-globuline et s'affaiblit notablement (voir les Figs. 1B et 1C). Une fusion partielle et un affaiblissement identique entre les arcs β_{2A} et γ-globulines sont signalés par HEREMANS *et coll.*[2], qui les rattachent à une identité antigénique partielle entre ces globulines. Cette identité antigénique partielle entre la β_{2A}-globuline et les γ-globulines gêne dans ces diagrammes l'analyse précise du point de raccordement des arcs de la globuline anormale et de la β_{2A}-globuline. Cette étude nécessite l'utilisation d'un sérum ne possédant plus les anticorps spécifiques des γ-globulines, mais révélant la β_{2A}-globuline. Deux immunsérums de ce type ont été mis en œuvre:

(a) Un antisérum préparé à partir du colostrum humain révèle l'arc β_{2A} du sérum humain normal. Il révèle également, mais plus faiblement, la globuline anormale du myélome β_2 étudié (Fig. 3B). Le sérum du malade enrichi en β_{2A}-globuline normale montre une identité antigénique entre ces deux globulines, dont les arcs se raccordent totalement (Fig. 3B). Ce même antisérum révèle très faiblement les γ-globulines normales, confirmant une identité antigénique partielle entre la β_{2A} et les γ-globulines normales.

(b) L'immunsérum humain épuisé par les protéides du sérum de cordon ombilical* révèle également la globuline anormale et montre en outre l'importance de cet arc de précipitation, qui se prolonge jusque dans la zone α_2, surcroisant l'arc de l'α_2-céruléoplasmine (identifié par une révélation spécifique, selon la technique préconisée par URIEL[11]). On note un renforcement localisé de son extrémité α_2 qui est plus concave. Le sérum de myélome enrichi en β_{2A}-globuline, révélé avec cet immunsérum, montre un renforcement de l'extrémité cathodique β_2, correspondant à la β_{2A}-globuline normale, qui se raccorde totalement avec la globuline anormale (Fig. 3 C).

RÉSUMÉ

Il existe dans le cas de myélome étudié une identité électrophorétique et immunologique totale entre la globuline trouvée dans les plasmocytes anormaux de la moelle et la globuline anormale identifiée dans le sérum.

La globuline anormale possède d'autre part des caractères physico-chimiques différents de la β_{2A}-globuline normale, qui se traduisent par une différence de vitesse électrophorétique. Cependant, l'identité antigénique des deux globulines est parfaite vis-à-vis de l'immunsérum antiglobuline β_{2A} obtenu avec le colostrum humain ou de l'antisérum épuisé par du sérum de cordon ombilical, qui ne possèdent que les anticorps spécifiques de la β_{2A}-globuline et sont débarrassés des anticorps des γ-globulines.

En effet, la globuline β_{2A} possède au moins deux déterminants antigéniques, dont l'un est commun avec l'un des déterminants antigéniques des γ-globulines et provoque la formation d'anticorps anti- γ-globulines normales, l'autre (ou les autres) étant spécifiques de la β_{2A}-globuline normale. *La globuline myélomateuse possède certainement*

* Le sérum du sang de cordon ombilical ne contient pas de céruléo-plasmine, d'haptoglobine, de β_{2A}, de β_2 ni de γ-globulines. L'immunsérum épuisé par les protéides sériques de sang de cordon ombilical révèle les arcs correspondant à ces globulines, ainsi que les β-lipoprotéines.

Bibliographie p. 700

la totalité de ces déterminants antigéniques β_{2A}. Il faut souligner cependant que seule l'utilisation d'un antisérum spécifique de cette globuline anormale pourrait nous apporter la preuve de l'absence de déterminant antigénique particulier.

SUMMARY

PLASMOCYTIC ORIGIN OF A MYELOMATIC β_2-GLOBULIN. INVESTIGATION OF ITS IMMUNOELECTROPHORETIC PROPERTIES IN COMPARISON WITH β_{2A}-GLOBULIN

In the case of myeloma studied the globulin found in the abnormal plasmocytes of the marrow and the abnormal globulin identified in the serum proved to be identical both electrophoretically and immunologically.

On the other hand, the physicochemical properties of the abnormal globulin differ from those of normal β_{2A}-globulin, as shown by their different electrophoretic mobilities. The two globulins are, however, completely identical as regards their antigenicity towards anti-β_{2A}-globulin immune serum obtained with human colostrum or antiserum exhausted with umbilical cord serum, which contain only the specific antibodies of β_{2A}-globulin and are devoid of γ-globulin antibodies.

Actually β_{2A}-globulin has at least two antigenic determinants, one of which is the same as one of the γ-globulin determinants and gives rise to the formation of the normal anti-γ-globulin antibodies, while the other (or others) are specific for normal β_{2A}-globulin. *The myelomatic globulin certainly possesses all these antigenic* β_{2A} *determinants.* It should be emphasized, however, that only by using a specific antiserum of this abnormal globulin will it be possible to prove that a particular antigenic determinant is absent.

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AN IMPROVED DIACETYLMONOXIME METHOD FOR THE DETERMINATION OF UREA IN BLOOD AND URINE

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The routine procedures used for the determination of urea in blood serum and urine vary with the facilities of the clinical laboratories. The procedure should be sufficiently sensitive and accurate for correct clinical interpretation but should also be simple in technique and inexpensive.

A considerable number of different procedures have been suggested for the determination of urea in biological fluids. These include methods involving titration, micro-diffusion, nesslerization, autoclave-hydrolysis, xanthydrol[1, 2], diacetyl[3], diacetylmonoxime[4–9], α-isonitrosopropiophenone and diazotization. The urease method is generally regarded as a standard procedure because it is most specific and reliable. However, the diacetyl and the diacetylmonoxime methods are simplest and are particularly suitable for routine work. Several years ago the diacetylmonoxime method was chosen in our laboratories and an attempt was made to assess its accuracy and reproducibility.

In principle the diacetylmonoxime method is a colorimetric application of FEARON'S[6] reaction which consists in the production of an oxidative yellow cyclic compound of urea and diacetyl. The yellow coloration may, however, fade particularly in the presence of the oxidizing substances required to eliminate the reductive inhibition exerted by hydroxylamine, which is an inevitable byproduct of the reaction. Various kinds and amounts of oxidizing agents have been tested for the development of a stable and satisfactory colour.

At first, NATELSON'S[3] procedure which uses a diacetyl reagent to circumvent the oxidizing agent was examined, but it was insufficiently reproducible. FRIEDMAN[7] had already pointed out the rapid discoloration of the yellowish hue in this reaction. The diacetylmonoxime method also failed to provide a simple reliable procedure, because it was very sensitive to the oxidizing agents with a considerable colour variation, and required meticulous preparation of the reagents. Investigations showed that perchloric acid was the most suitable oxidant, while sulfuric acid was chosen as an adequate solvent for diacetylmonoxime; it not only prevented the offensive smell produced during heating, but also permitted colorimetry without dilution of the solution. It was also shown that obedience to Beer's law was not essential for the exact determination of urea.

The proposed diacetylmonoxime method therefore employs sulfuric acid and perchloric acid. It compares favorably with the urease method for high concentrations of urea, although it may be somewhat less accurate for lower concentrations (less than 10 mg/100 ml). The calibration curve is reliable up to the level of 100 mg/100 ml. The procedure is sufficiently simple for routine work.

References p. 706

EXPERIMENTAL

Reagents

1. Trichloroacetic acid solution: 10 g of trichloroacetic acid are dissolved in 100 ml of water.

2. Diacetylmonoxime solution: 1.0 g of diacetylmonoxime is dissolved in 100 ml of 5% acetic acid. It keeps for several months.

3. Perchloric acid–sulfuric acid mixture: 60 ml of concentrated sulfuric acid are added dropwise into 240 ml of water which is kept cool. To this are added 22 ml of perchloric acid (70%).

Procedure

1. 0.1 ml serum is placed in a test tube, to which are added 1.5 ml water and 1.4 ml trichloroacetic acid solution. The mixture is filtered on paper or centrifuged to give a clear filtrate or supernate.

2. An aliquot of 1.0 ml of the filtrate or supernate is transferred to a stoppered test-tube; 1 ml of diacetylmonoxime solution and 4.0 ml of perchloric acid–sulfuric acid mixture are added, and the stoppered test-tube is treated in a boiling water bath for 30 min.

3. The test-tube is then removed and cooled in running water. The optical density of the solution in the test-tube is measured in a photoelectric colorimeter at a wavelength of 470 mμ against a blank consisting of distilled water. The concentration of urea is read from the calibration curve constructed as stated below.

Colorimetry should be done immediately after the removal of the test-tube from the bath, because the color may fade rapidly under direct sun-light, though it is not affected by scattered light.

For the determination of urea in urine, water should first be added to the urine so that the solution contains 10–100 mg of urea/100 ml; the diluted solution is then treated as described above.

Calibration curve

A standard aqueous solution of urea (30 mg/ml) is diluted 200 times with water to obtain the working standard solution. Aliquots of 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4 ml of this solution are diluted to 3.2 ml with distilled water to give dilutions corresponding to 7.5, 15.0, 22.5, 30.0, 45.0, 60.0, 75.0, 90.0 and 105.0 mg of urea/100 ml of serum; 2.8 ml of trichloroacetic acid are added to each aliquot and mixed. One ml portions of each mixture are treated with diacetylmonoxime reagent and perchloric acid–sulfuric acid mixture, and their optical densities are measured as in 2. and 3. above. A calibration curve, which has a sigmoid form (Fig. 1), is constructed from the optical densities and the urea concentrations.

DISCUSSION

The coloured solution had an absorption maximum at 480 mμ as in other diacetylmonoxime methods. However, the relationship of optical density to urea concentration was not rectilinear at this wavelength; similar sigmoid calibration curves were also obtained over the range 460–490 mμ. Attempts to discover the conditions under which Beer's law is obeyed were less successful than those to obtain a stable calibration curve, possibly because the colour results from the complicated

References p. 706

interaction of numerous factors participating in FEARON's reaction. The sigmoid calibration curve was not an obstacle to colorimetry when ordinary photoelectric colorimeters were used.

FEARON's reaction usually proceeds in a strong hydrochloric acid medium, which exerts a considerable influence on the resultant coloration. The effect of acidity was

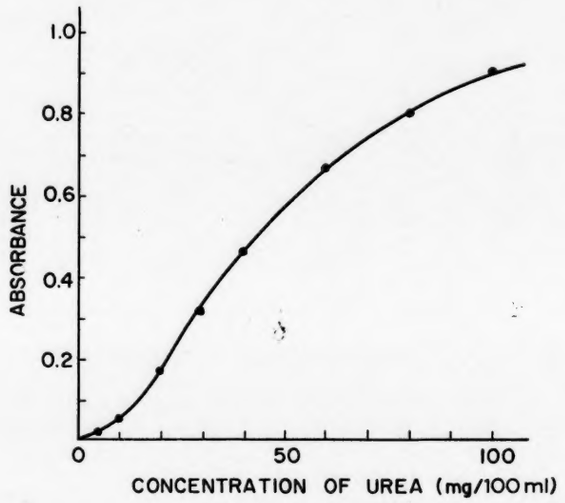

Fig. 1. Calibration curve.

similar but less marked when sulfuric acid was employed as a medium. The maximum coloration was obtained over the range of acidity from 4 N to 6 N (Fig. 2), and the color intensity did not vary so much that strict control of acidity was essential; 5 N sulfuric acid was therefore chosen as the most suitable solvent.

The concentration of oxidant exerts a considerable influence on the intensity of color produced in FEARON's reaction, and perchloric acid was no exception in this respect. Perchloric acid seems to promote the discoloration of the final color although

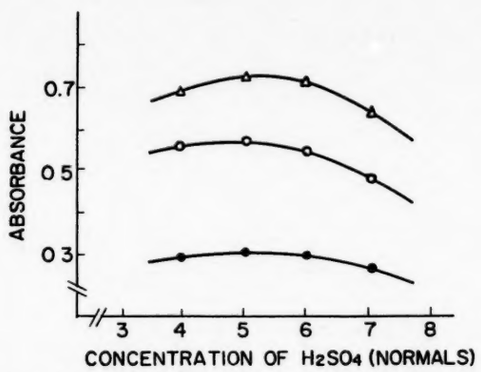

Fig. 2. Dependence on sulfuric acid concentration.
Urea: –●– 7 μg, –○– 13 μg, –△– 23 μg.

it has a beneficial effect on the reaction by the elimination of hydroxylamine. The optimum concentration of perchloric acid had, therefore, to be determined so that the maximum elimination of hydroxylamine might be attained with the minimum

References p. 706

discoloration; for this purpose, the amount of urea in the fluid to be analysed had to be considered. Fig. 3 shows that the optimum concentrations of perchloric acid for the maximum coloration vary with the amounts of urea. Perchloric acid concentrations of 1.5–2% and of 3–4% were adequate for 3–10 μg and 20–30 μg of urea, respectively. However, the variation was less pronounced than when arsenic acid

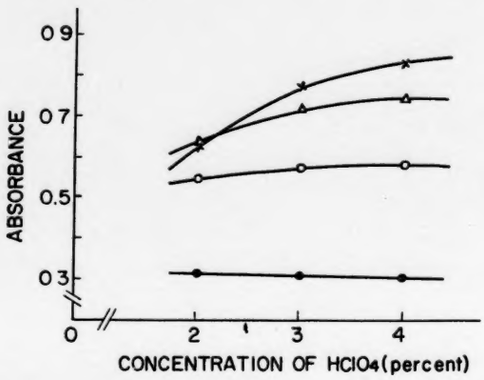

Fig. 3. Dependence on perchloric acid concentration.
Urea: –●– 10 μg, –○– 16 μg, –△– 23 μg, –×– 33 μg.

was used as oxidant. The final concentration of 3.4% of perchloric acid was chosen in order to obtain approximately maximum colorations over as wide as possible a range of urea concentrations, namely 0–120 mg/100 ml. With less concentrated perchloric acid, the color decreased remarkably in intensity for higher concentrations of urea although there was a slight increase for lower concentrations. The chosen concentration of perchloric acid was also adequate when hydrochloric acid was used as solvent[10].

The coloration increased on heating, and reached a maximum after 1 h in a boiling water bath (Fig. 4). Heating for 10 min may be sufficient if care is taken with regard to exactitude in timing and to the sensitivity of the colorimeter. In the present procedure heating for 30 min is specified for convenience in routine work.

The calibration curve was sigmoid in shape with an approximately rectilinear portion for the range of urea from 10–60 mg/100 ml; in this range, the urea concen-

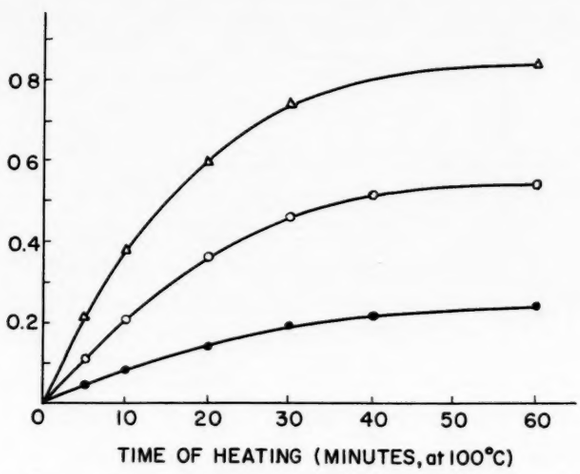

Fig. 4. Time curve. Urea contents: –●– 7 μg, –○– 13 μg, –△– 23 μg.

References p. 706

trations in the samples were read with accuracy, but the linear relationship between absorbance and concentration was not observed below 10 mg/100 ml or over 70 mg/100 ml. Construction of calibration curves was, therefore, necessary in every determination.

The reagents were renewed several times to check the stability of the calibration curve; the optical densities of each set of urea solutions agreed quite well within the limit of experimental error. The calibration curves were also essentially the same when they were made in duplicate. Distilled water proved to be satisfactory as a blank.

Errors from the calibration curve were negligible with urea concentrations of 10–60 mg/100 ml; the accuracy was slightly less below 10 mg and above 70 mg/100 ml. Preliminary dilution is recommended for samples containing over 100 mg of urea/100 ml.

All the steps of the procedure were conducted in a laboratory facing south and direct sunlight was avoided by means of a Venetian blind. The color faded with increasing rapidity with decrease in urea concentrations. It faded slowly when direct sunlight was avoided; the optical density decreased by 20–30%, 5% and about 1%, for samples with urea concentration of less than 5 mg, 5–20 mg and over 60 mg/100 ml, respectively, after 2 h exposure to diffuse light in the laboratory. No appreciable decoloration occurred in samples of higher urea concentration. Dark glass test tubes were therefore unnecessary when the colored solutions were subjected to colorimetry 10 to 15 min after the completion of development.

The specificity of this reaction is essentially similar to that of the carbamide diacetyl reaction investigated by FEARON[6]. Some drugs that are carbamide derivatives interfere with this reaction, *e.g.* phenacetylurea, sulfanilyl-*n*-butylurea (Bz-55), thiosemicarbazone, adrenochrome semicarbazone, bromisovalerylurea, and carbarsone. In actual fact the errors arising from these drugs may be negligible. Tolylsulfonyl-*n*-butylurea (D-860), barbiturate, diphenyl-hydantoin, isonicotinylhydrazide, and aminophylline do not interfere.

Duplicate determinations with samples containing 10–55 mg of urea/100 ml showed that the discrepancy was less than 0.4%.

TABLE I

RECOVERY TEST

Serum	*Urea added mg/100 ml*	*Original urea concn. mg/100 ml*	*Calculated urea concn. mg/100 ml*	*Recovery %*
1	0	16.7	—	—
	15	31.6	31.7	99.7
	30	45.3	46.7	97.0
	60	74.4	76.7	97.3
2	0	37.0	—	—
	15	52.6	52.0	101.0
	30	65.3	67.0	97.9
	60	93.2	97.0	96.2
3	0	175.4	—	—
	15	188.1	190.4	98.9
	30	204.3	205.4	99.5
	60	234.0	235.4	99.5

References p. 706

The recovery of urea by the present procedure was 98.6 ± 1.4% (Table I). There was a satisfactory agreement between the urease-nesslerization method and the present method (Table II).

TABLE II
COMPARISON WITH THE UREASE-NESSLERIZATION METHOD

Serum	*Present method mg of urea/100 ml*	*Urease method mg of urea/100 ml*	*Urine*	*Present method g of urea/100 ml*	*Urease method g of urea/100 ml*
1	11.0	10.6	1	0.10	0.08
2	12.9	13.2	2	0.31	0.30
3	13.0	11.7	3	0.44	0.44
4	15.0	15.7	4	0.53	0.50
5	16.5	16.0	5	0.66	0.64
6	30.0	29.1	6	0.73	0.65
7	44.0	43.3	7	1.06	1.00
8	100.0	100.0	8	1.64	1.84
9	167.0	162.0	9	4.23	4.26

ACKNOWLEDGEMENT

We wish to acknowledge our gratitude to Prof. Dr. S. SHIBATA, Head of the Department of Clinical Pathology of Yamaguchi Medical College for his support.

SUMMARY

An improved procedure for the determination of urea in blood serum and urine is presented. Blood serum is deproteinized with trichloroacetic acid and the resulting filtrate heated for 30 min with diacetylmonoxime reagent in presence of perchloric acid to develop a stable yellowish color. The colored solution is measured at 470 mμ.

The procedure is satisfactory in routine work, being simple and reliable. It compares quite well with the conventional urease method.

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A NEW METHOD FOR THE QUANTITATIVE DETERMINATION OF HAPTOGLOBIN

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Two methods have been described in the literature for the quantitative determination of serum haptoglobin. In the first method[1], the peroxidase activity of the haemoglobin–haptoglobin complex is measured in the system:

$$C_2H_5OOH + 2I^- + 2H^+ \rightarrow C_2H_5OH + I_2 + H_2O$$

after the excess of haemoglobin, which is added to the serum, has been inactivated by iodine. The second method[2] depends on the difference in electrophoretic mobility of the haptoglobin–haemoglobin complex and free haemoglobin at pH 7.0. The largest amount of haemoglobin that can be bound by the haptoglobin in a given amount of serum, without any free haemoglobin being detectable after electrophoresis, is determined. Both methods have disadvantages. In the first method a blank has to be determined; the second method is time-consuming because it is not known in advance how much haemoglobin must be added to the serum.

In this paper a method is described which avoids both disadvantages. The principle of the method is to separate the excess of haemoglobin from the haptoglobin–haemoglobin complex by paper electrophoresis at pH 6.9. The free haemoglobin migrates to the anode, and the complex to the cathode. The amount of complex can be determined by elution of the complex from the paper, followed by a spectrophotometric assay of peroxidase activity.

MATERIALS AND METHODS

Bovine haemoglobin solutions were prepared by washing an erythrocyte suspension several times with 0.9% sodium chloride. An equal volume of distilled water and half a volume of toluene were added, and the mixture was shaken vigourously for 2 min. After standing for 14 h at 2°, the mixture was centrifuged, toluene and stroma proteins were siphoned off and the haemoglobin solution was filtered through Celite 535. An aliquot was taken for a concentration determination by the cyan–methaemoglobin method, using a millimolar extinction coefficient of 11.4 (ref.[3]).

The remainder of the haemoglobin was converted into carboxyhaemoglobin by passing a rapid stream of carbon monoxide through the solution.

Excess haemoglobin (Hb–CO) was added to a serum sample and 0.03 ml of this mixture was applied to the middle of a strip of moistened filter paper (Whatman No. 1), 5 cm broad, on a line 3 cm long. The paper electrophoretic separation was carried out with a 0.05 *M* sodium phosphate buffer pH 6.9, overnight for 14 h at a potential gradient of 2 V/cm at 2°.

After electrophoresis, a band of 2 cm from the starting line to the cathode was

References p. 709

cut out and eluted by the method of SANGER AND TUPPY[4]. For this purpose, a tip was cut in the margin of the paper that was not used for the electrophoresis.

A small edge of the paper was fixed between two glass slides (2 × 3 cm), the whole was placed in a trough filled with phosphate buffer pH 6.9, and the eluate was collected in a capillary, 20 × 0.5 cm (see Fig. 1).

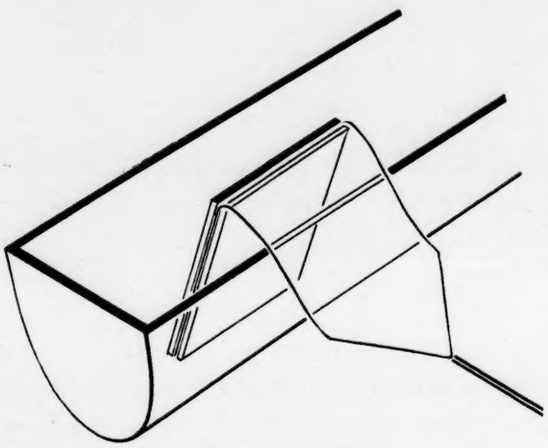

Fig. 1. The elution of the complex from the paper.

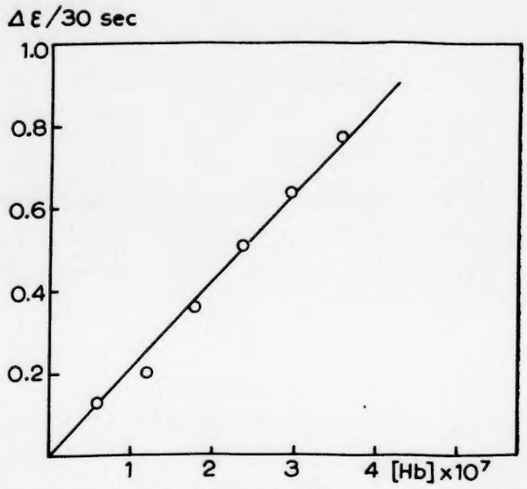

Fig. 2. Illustration of the linear relationship between initial rates of reaction and enzyme concentration.

Peroxidase activity was measured by a slight modification of the method described by CHANCE AND MAEHLY[5].

The system consisted of 0.15 *M* sodium acetate buffer pH 5.0, and 20 mmoles of guaiacol. Just before use, a definite amount of concentrated hydrogen peroxide solution was added to bring the concentration up to 40 mM. Optical densities were read at 470 mμ, using a Unicam spectrophotometer model SP 500.

When the elution was complete (3 h) the content of the capillary was blown into a cuvette which contained 3 ml of the guaiacol solution, and a stopwatch was started. After 30 sec the optical density was read.

RESULTS AND DISCUSSION

Conditions for the measurement of peroxidase activity were chosen so that a linear relationship was obtained between the initial rates of reaction (*i.e.* the $\triangle\varepsilon$ for the first 30 sec of the reaction) and the concentration of enzyme, *i.e.* haemoglobin or haptoglobin–haemoglobin complex; Fig. 2 shows that such a relationship in fact exists.

In Fig. 3a the results are given of the paper electrophoretic separation of haemoglobin and haptoglobin–haemoglobin complex in three different normal sera. The electropherograms were stained with benzidine according to the procedure of OWEN, SILBERMAN AND GAT[6]. The resulting colour, when kept in the dark, was stable for months. Fig. 3b gives the result obtained with haemoglobin alone. No peroxidase activity could be found at the cathode side of the starting line.

After elution of the haemoglobin–haptoglobin complex the paper was placed in an aqueous solution of benzidine in sodium acetate buffer pH 4.7, to which hydrogen peroxide was added. Only after 2 min a very faint blue coloration was visible, indicating that the elution of the complex was practically complete. In order to judge the

References p. 709

reproducibility of the method, four runs of the same serum were made. For $\triangle\varepsilon \times 10^3$/30 sec we found 216, 223, 215 and 221 respectively, with a mean of 219 and a mean error of 4, *i.e.* 1.8%. This is sufficient for clinical purposes.

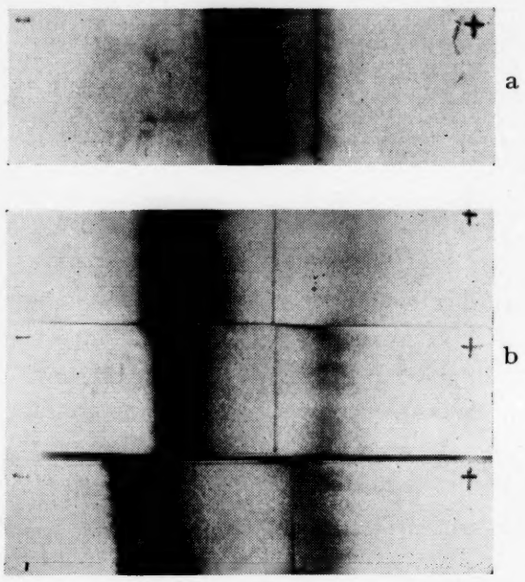

Fig. 3. Paper electrophoretic separation of haemoglobin and haptoglobin–haemoglobin complex.

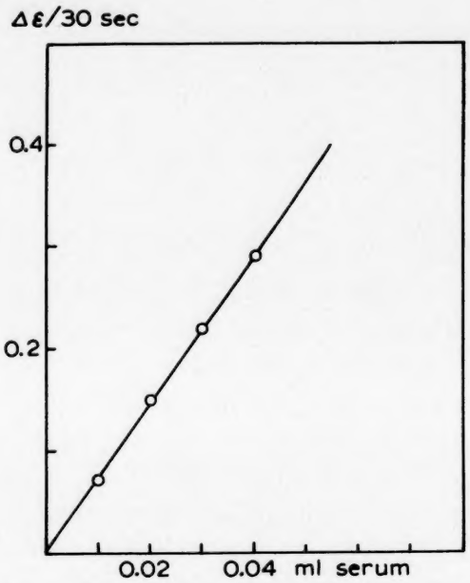

Fig. 4. Relationship between the amount of serum and peroxidase activity.

Excess of haemoglobin did not interfere with the determination. To the same serum sample increasing amounts of haemoglobin were added from 240 mg Hb per 100 ml serum to 1220 mg Hb per 100 ml serum in five steps. Within the experimental error, all five specimens gave the same results.

As shown in Fig. 4, a linear relationship exists between the peroxidase activity and the amount of serum applied to the paper as would be expected.

Normal values of haptoglobin levels lay between a $\triangle\varepsilon \times 10^3$/30 sec of 26 and 220 with a mean of 165, as determined in duplicate in 15 serum samples of normal healthy people.

ACKNOWLEDGEMENT

The aid of Dr. C. A. M. HAANEN in the collection of serum samples is gratefully acknowledged.

SUMMARY

A method is described for the quantitative determination of haptoglobin by paper electrophoretic separation of haemoglobin and haptoglobin–haemoglobin complex, followed by spectrophotometric assay of the peroxidase activity of the latter.

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THE DETERMINATION OF THYROXINE LEVELS IN HUMAN PLASMA BY DOUBLE ISOTOPE-DILUTION TECHNIQUE

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INTRODUCTION

The determination of thyroxine and its analogues in biological materials has always presented some difficulty owing to the extremely small amounts of the compounds present except in the thyroid gland itself. Bioassay methods used in the assay of thyroid hormones in thyroid therapy preparations are much too insensitive for adaptation for the determination of these substances in serum[1-5]. The nitrous acid colorimetric test[6, 7] has been used for the semi-quantitative determination of thyroxine in plasma from thyrotoxic patients[8], but the method is non-specific for the iodothyronines and also lacks the sensitivity for the estimation of plasma levels of subjects of normal and hypo-thyroid function. A similar lack of sensitivity is exhibited in the colorimetric test using diazotised N'-diethylsulphanilamide[9], although this reaction is more specific for the thyroid hormones.

In recent years the assessment of the level of iodothyronines in blood plasma has relied on the determination of the organic iodine associated with the serum proteins (Protein-Bound Iodine or PBI)[10, 11]. PBI determination has proved a useful diagnostic test for thyroid function, but it is also non-specific and its value is completely destroyed if the patient had ingested any iodine-containing compounds[11].

The method described in this paper is an extension of the general technique of amino acid determination by double isotope dilution using tritiated acetic anhydride as an acetylating reagent described by WHITEHEAD[12]. The free amino acids including thyroxine and its analogues in the sample are acetylated with acetic anhydride labelled with [^{3}H] of high specific activity. A known amount of acetyl thyroxine labelled with [^{14}C] is added and a pure specimen of the derivative is isolated. The amount of thyroxine can be calculated from a determination of the [^{3}H] and [^{14}C] contents of the isolated material. The method appears to be reproducible to ± 2–3%, and the plasma thyroxine levels of a number of human subjects with normal and abnormal thyroid function are given.

EXPERIMENTAL

Materials and methods

Preparation of [1-^{14}C] acetyl-DL-thyroxine

This compound was prepared by hydrolysis of NO-[1-^{14}C]-diacetyl-DL-thyroxine ethyl ester made by a slight modification of the method of ASHLEY AND HARINGTON[13] for the preparation of the inert compound.

60 mg DL-thyroxine (Roche Products Ltd.) was dissolved in 0.1 *N* alcoholic sodium hydroxide (4.5 ml) in a stoppered tube. (1-^{14}C) acetic anhydride (100 μC/mmole)

References p. 720

solution in redistilled toluene (5 mg/ml, 2 ml) was added and the contents of the tube shaken. Three further additions of 0.1 *N* alcoholic sodium hydroxide (2 mls) and the labelled reagent (2 ml) were made at intervals of 10 min with vigorous shaking between additions. The mixture was allowed to stand for 1 h at room temperature and overnight in the refrigerator. The solution was transferred to a 250-ml flask, 5 ml of 6 *N* sulphuric acid added and the toluene and alcohol removed in vacuo below 40° C. The NO-[1-^{14}C]-diacetyl-DL-thyroxine ethyl ester which separated out as a white solid was centrifuged down and washed by centrifugation with water. The labelled ester was purified by taking up in ethanol (5 ml) and pouring the solution into boiling 0.2 *N* sulphuric acid (40 ml). After standing in the refrigerator overnight the precipitate was centrifuged and washed with water.

The NO-[1-^{14}C]-diacetyl-DL-thyroxine ethyl ester was taken up in *N* sodium hydroxide solution in aqueous ethanol (50% v/v 1 ml). After standing at room temperature, the solution was made acid with *N* HCl and 10 ml water was added. The N-[1-^{14}C]-acetyl-DL-thyroxine which separated as a white precipitate was centrifuged and washed with water.

The product was tested chromatographically for isotopic purity and a standard solution (1 mg/ml) in ethanol and 2 *N* aqueous ammonia (1 : 1 v/v) was prepared and stored in a glass-stoppered tube in the deep freeze cabinet.

Tritiated acetic anhydride

This compound was prepared from tritiated acetic acid produced by the decarboxylation of malonic acid which had been allowed to exchange with tritiated water[12]. A stock solution of tritiated acetic anhydride of specific activity of 60 mC/mmole in redistilled toluene (5 mg/ml) was used as the analytical reagent.

Standardisation of tritiated acetic anhydride reagent

The equivalent specific activity of the tritiated reagent was given by a determination of the specific activity of a pure sample of hydrocortisone acetate prepared with the labelled acetic anhydride[14].

Preparation of samples for analysis

Serum samples were obtained by centrifuging the whole blood without any additions within 30 min of the taking of the samples from the subjects. The serum was stored in deep freeze until the analysis could be carried out.

Calcium acetate solution

A. R. glacial acetic acid (10 ml) was added to a suspension of calcium hydroxide (7 g) in water (50 ml). The resulting solution was made up to 200 ml with water and after filtration was pH 6.

Chromatography solvents

(*a*) *n*-Butanol–methanol–aq. 2 *N* NH_3 (4 : 1 : 5 by vol.) (two phases).
(*b*) Ethyl acetate–methanol–aq. 2 *N* NH_3 (4 : 1.5 : 1.9 by vol.) (two phases).

Diazotised sulphanilic acid solution

This reagent was prepared by adding together equal volumes of alcoholic hydrochloric acid (ethanol–conc. HCl, 9 : 1 by vol.), amyl nitrite in ethanol (10% v/v)

References p. 720

and a solution of the sodium salt of sulphanilic acid (1% w/v in ethanol–water, 1 : 1 by vol.), allowing to stand for five minutes or until the white diazonium salt separated. An equal volume of water was then added to clear the solution.

General procedure for the determination of thyroxine using [^{14}C] and [^{3}H]

The sample of serum (5 ml) was pipetted into a R.B. flask of 100-ml capacity carrying a B. 19 neck. 2 *N* NaOH (*ca.* 0.2 ml) was added to bring the pH of the serum above 9. The mixture was acetylated using five successive additions of standardised tritiated acetic anhydride reagent (0.2 ml) and five additions of 0.1 *N* NaOH (0.2 ml) with the apparatus and procedure described by WHITEHEAD[12]. The serum was covered with ether (5 ml) to prevent frothing during the acetylation. After the acetylation an aliquot of standard [^{14}C] N-acetyl thyroxine (approx. 100 μg and approx. 10,000 counts/min) was added, the delivery tube removed from the flask after washing with a minimum of water, and the flask attached by the B. 19 neck to a high-vacuum manifold. The solution was taken down to approximately 2 ml *in vacuo*. Acetic acid (10% v/v) was added to pH 6 followed by absolute ethanol (7.5 ml) to complete the precipitation of serum proteins. The solution and most of the precipiate were transferred to a centrifuge tube (50-ml capacity). After centrifugation at 0°, the clear supernatant liquid was poured into a clean stoppered centrifuge tube. Water (3 ml) was added to the flask in which the acetylation had been carried out, the contents shaken and transferred to the tube containing the protein residue. After stirring with a glass rod, absolute ethanol (7.5 ml) was added and the protein again centrifuged down. The second extract was poured into a clean stoppered tube.

Aqueous 2 *N* NH_3 (1 ml) was added to each extract which was then shaken with chloroform (5 ml). The tubes were spun in the centrifuge at 0° C to complete the separation of the solvent layers. The chloroform layers were removed by a suction pipette, extracted separately with the same aliquot of water (5 ml) and then discarded. The aqueous extracts were bulked into one tube for each sample.

Calcium acetate solution (2 ml) was added to the combined extracts from each sample to precipitate any remaining fatty acids. The precipitate was centrifuged to the bottom of the tube. The supernatant solution was passed through an ion exchange resin column (1 cm diameter) prepared from 3 g Zeokarb 225 in the acid condition and suspended in ethanol–water (1 : 1 v/v). Any residue remaining in the centrifuge tube was washed onto the column with the ethanol–water mixture (2 × 2 ml). Ethanol–aqueous NH_3 (1 : 1 v/v) was put through the column until the darker-coloured front just reached the bottom of the column. The combined eluate was made alkaline to pH 9 with aqueous 2 *N* NH_3 and evaporated to dryness *in vacuo*.

The residue was taken up in water (4 ml) and acetic acid (1 ml 10% v/v) was added. The solution was extracted with one 10-ml portion followed by three 5-ml portions of *n*-butanol–chloroform (30% v/v, half saturated with water). The chloroform layers were bulked and taken to dryness *in vacuo*.

The residue was taken up in ethanol–aqueous 2 *N* NH_3 (0.2 ml 1 : 1 v/v), applied to Whatman No. 2 filter paper, then run up to the origin in acetone–2 *N* aqueous NH_3 (1 : 1 v/v) by the technique described by BUSH[15]. A two-dimensional chromatogram was then run in *n*-butanol–methanol–aqueous 2 *N* NH_3 and ethyl acetate–methanol–aqueous 2 *N* NH_3[12]. The acetyl thyroxine was shown up by spraying with aqueous sodium carbonate (5% w/v) followed by diazotised sulphanilic acid.

References p. 702

Measurement of radioactivity

The area of paper (approx. 1–2 cm^2) containing the labelled N-acetyl thyroxine was cut out from the developed chromatogram and burnt in a stream of oxygen. The [^{14}C] and [^{3}H] contents of the carbon dioxide and water so produced were determined by gas-counting technique[12, 14]. The thyroxine present in the sample of serum was calculated using the expression (1) given below.

Determination of the specific activity of [^{131}I]-labelled thyroxine

A suitable aliquot of the [^{131}I]-labelled thyroxine solution (= approx. 0.5 μg) was acetylated in the usual way with standardised tritiated acetic anhydride[12]. A known quantity of [^{14}C]-labelled acetyl thyroxine was added to the acetylated solution which was then passed through Zeocarb 225 in a column. The active acetyl thyroxine in the eluate was separated on a two-dimensional chromatogram run in *n*-butanol–methanol–aqueous 2 *N* NH_3 and ethyl acetate–methanol–aqueous 2 *N* NH_3. The acetyl thyroxine spot was cut from the paper and the [^{131}I] activity was counted by placing the piece of paper in the well of a scintillation counter. The paper was then combusted and the [^{14}C] and [^{3}H] content measured by gas counting. Silver wire was inserted in the end of the combustion tube to prevent any active iodine from reaching the liquid nitrogen traps on the combustion train. The specific activity of the [^{131}I]-labelled thyroxine was calculated by expression (2) below.

The radioactive purity of the sample was checked by applying the same aliquot as was taken for the determination of the specific activity of the thyroxine to a piece of Whatman No. 2 paper (2 cm^2) and counting in the scintillation well counter. The percentage purity was calculated from expression (3) below.

Determination of thyroxine in serum using [^{131}I] and [^{3}H]

(*a*) *Estimation without extraction.* [^{131}I]-labelled thyroxine (0.01 μg, approx. 30,000 counts min) was added to an aliquot of the serum (5 ml). The serum was acetylated with standard tritiated acetic anhydride and the N-acetyl thyroxine isolated by the general procedure. The [^{131}I] activity was determined in the scintillation well counter and the tritium activity by combustion and gas counting in the usual way. An aliquot of the [^{131}I]-labelled thyroxine (0.01 μg) was applied to paper and counted at the same time as the sample. The amount of thyroxine in the sample of serum was given by the expression (4) below.

(*b*) *Estimation with extraction.* After the addition of [^{131}I]-labelled thyroxine (0.01 μg approx. 30,000 counts/min) the sample of serum (5 ml) was adjusted to pH 2–3 with *N* sulphuric acid and extracted with *n*-butanol equilibriated with dilute sodium thiosulphate solution (2 × 12.5 ml). The combined extracts were made alkaline with methanol–aqueous 5 *N* NH_3 (4 : 1, v/v) and evaporated to dryness *in vacuo*[16]. The extract was taken up in 2 *N* NaOH (0.4 ml), the volume adjusted to (5 ml) with water, acetylated with standard tritiated acetic anhydride and the acetylated mixture processed in the usual way. The [^{131}I] and [^{3}H] activities of the isolated *N*-acetyl thyroxine were determined as described under (a).

Hydrolysis of serum

A number of analyses were carried out on aliquots of the same serum after hydrolysis by different procedures[17].

References p. 720

(*a*) 5 ml serum was made alkaline (pH 8.4) with 0.1 *N* NH_3, ammonium chloride buffer (1.0) ml, crystalline trypsin added and the mixture incubated in a stoppered tube at 37° for 72 h. Further additions of buffer (0.5 ml) and trypsin (3 mg) were made after each 24 h.

(*b*) 5 ml serum was heated at 95–100° with 2 ml baryta (8%, w/v) for 8 h.

Isotope dilution equations

If If H_a = Equivalent activity of the tritiated acetic anhydride (counts/min/μequiv).
H_b = Final [^{3}H] activity in the separated N-acetyl thyroxine (counts/min).
C_a = Activity of [^{14}C]-labelled N-acetyl thyroxine added (counts/min).
C_b = Final [^{14}C] activity in the separated N-acetyl thyroxine (counts/min).
I_a = Activity of [^{131}I]-labelled thyroxine added (counts/min).
I_b = Final [^{131}I] activity in separated N-acetyl thyroxine.
m = Micromolecular weight of thyroxine.
w = Weight of thyroxine in the sample.
x = Weight of [^{131}I]-labelled thyroxine added.
P = Isotopic purity of [^{131}I]-labelled thyroxine.

then in the determination of thyroxine using [^{3}H] labelled reagent and adding [^{14}C]-labelled N-acetyl thyroxine,

$$w = \frac{C_a \cdot H_b \cdot m}{C_b \cdot H_a} \mu g \tag{1}$$

The specific activity of the [^{131}I]-labelled thyroxine is given by the expression,

$$\frac{H_a \cdot C_b \cdot I_b}{H_b \cdot C_a \cdot m} \text{ counts/min/}\mu g \tag{2}$$

The radioactive purity of the [^{131}I]-labelled thyroxine is given by the equation,

$$P = \frac{I_b \cdot C_a \cdot 100}{I_a \cdot C_b} \text{ per cent} \tag{3}$$

The amount of thyroxine in the serum sample in the determination using [^{131}I]-labelled thyroxine and [^{3}H]-labelled reagent is given by the expression,

$$w = \frac{I_a \cdot H_b \cdot m \cdot P}{I_b \cdot H_a \cdot 100} - x \, \mu g \tag{4}$$

RESULTS

Fig. 1 shows the two-dimensional chromatogram obtained with mixtures of the N-acetyl derivatives of the iodo-tyrosines, thyroxine and its analogues and the other naturally occurring amino acids when run in *n*-butanol–methanol–aqueous 2 *N* NH_3 and ethyl acetate–methanol–aqueous 2 *N* NH_3. The abbreviations for the names of the amino acids shown in the figure are those used by BRAND AND EDSALL[18], and PITT-RIVERS[19].

The general method using [^{14}C]-labelled acetyl thyroxine and [^{3}H] acetic anhydride was tested by carrying out the procedure on aliquots of bulked samples of serum with and without additions of known quantities of thyroxine. The results obtained with three different sets of analyses are shown in Table I. Close agreement was found in each series between the thyroxine value obtained for the serum without addition of thyroxine and the sera thyroxine values deduced from the total thyroxine present in the aliquots to which thyroxine had been added. The standard deviation from the mean value for the thyroxine content of the original serum was 2–3%.

References p. 720

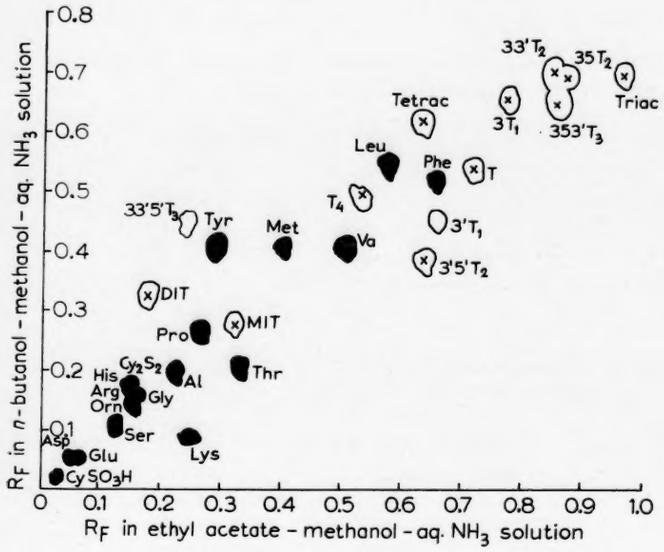

Fig. 1. Two-dimensional chromatogram of N-acetyl amino acids run in *n*-butanol –methanol–aq. NH_3 soln. (Vertical axis) and ethyl acetate–methanol–aq.NH_3 soln. (Horizontal axis). The abbreviations for the names of the commonly occurring amino acids other than the iodo-amino acids (shown in outline) are those used by BRAND AND EDSALL[18]. The abbreviations used for the iodo-amino acids (shown in black) are those used by PITT-RIVERS[19]. The two spots marked TRIAC and TETRAC refer to the O-acetyl derivatives of 3,5,3′-tri-iodo-thyro-acetic acid and tetra-iodo-thyro-acetic acid. The spot marked T refers to the N-acetyl derivative of unsubstituted thyronine.

The results of analyses for the thyroxine content of the same bulked serum by the general method and by the alternative method of adding a known amount of [^{131}I]-labelled free thyroxine before acetylation with the tritiated reagent are given in Table II. The values obtained by the different procedures were in close agreement showing a standard deviation from the mean of $\pm$ 3.2%.

The results obtained for the thyroxine content of human blood serum before and after hydrolysis by two different procedures are given in Table III. The values obtained from the untreated sample and the trypsin hydrolysate were the same within the experimental error of the method. Hydrolysis with baryta yielded a low thyroxine value. BRAASCH *et al.*[17] obtained a similar loss in experiments on known amounts of thyroxine treated in the same way. These results would appear to indicate that no thyroxine exists in blood serum which is combined by a peptide or other linkage which can be broken by the hydrolytic procedures used.

TABLE I

VALUES OBTAINED FROM ANALYSES OF SAMPLES OF HUMAN BLOOD SERUM TO WHICH KNOWN QUANTITIES OF THYROXINE HAD BEEN ADDED

Serum	*Volume of sample (ml)*	*Thyroxine added (μg)*	*Total thyroxine found (μg)*	*Thyroxine originally present (μg)*
A	5	0.254	0.468	0.214
A	5	0.127	0.367	0.240
A	5	0.0	0.236	0.236
				Mean 0.227 $\pm$ 3.1% (S.D.)
B	5	0.508	0.947	0.439
B	5	0.254	0.650	0.396
B	5	0.0	0.428	0.428
				Mean 0.421 $\pm$ 1.9% (S.D.)
C	5	0.508	0.860	0.352
C	5	0.381	0.719	0.337
C	5	0.254	0.617	0.363
C	5	0.127	0.476	0.349
C	5	0.0	0.376	0.376
				Mean 0.356 $\pm$ 1.8% (S.D.)

References p. 720

TABLE II

VALUES OBTAINED FROM ANALYSES OF THE SAME BULKED SAMPLE OF HUMAN BLOOD SERUM USING ALTERNATIVE ISOTOPE-DILUTION PROCEDURES

Method of analysis	*Thyroxine found (μg/100 ml)*
1. Determination by the acetylation of the serum with tritiated acetic anhydride followed by the addition of [^{14}C]-labelled acetyl thyroxine	6.10
2. Determination by the addition of [^{131}I]-labelled thyroxine followed by acetylation of the serum with [^{3}H]-labelled reagent	6.90
3. Determination by the addition of [^{131}I]-labelled thyroxine and the acetylation of the *n*-butanol extract with [^{3}H]-labelled reagent	6.28
	Mean 6.43 ± 3.2% (S.D.)

TABLE III

VALUES OBTAINED OF THE THYROXINE CONTENT OF HUMAN BLOOD SERUM BEFORE AND AFTER HYDROLYSIS BY DIFFERENT PROCEDURES

Hydrolysis procedure	*Thyroxine found (μg/100 ml)*	*Thyroxine destroyed during hydrolysis %*
(a) Trypsin (pH 8.4) at 37° for 72 h	5.3	nil
(b) 8% Baryta at 95° for 8 h	4.6	9
(c) Unhydrolysed serum	5.0	

The thyroxine levels in sera from 32 subjects are shown in Fig. 2. The degree of thyroid function as assessed from clinical examination, [^{131}I] tests, and other thyroid function tests carried out on the same patients is shown in the diagram by means of different shadings. Although precise ranges for the thyroxine levels cannot be fixed from this limited number of results, the indications are that normal subjects show a lower level of 3.0 μg/100 ml and an upper level of 6.5 μg/100 ml. Patients suffering from thyroid hypofunction fall within the range 0–3.5 μg/100 ml, and thyrotoxic patients show serum levels greater than 6.5 μg/100 ml.

The values for the serum thyroxine levels calculated in terms of iodine (μg/100 ml) are given in Table IV to facilitate a direct comparison with the serum protein-bound iodine (PBI) values. In all cases except two the thyroxine iodine values are lower than the PBI values. The two exceptions (sera 7 & 8) are both cases where the amounts of iodine involved are small and the errors of the two experimental techniques could account for the discrepancy. Values for protein-bound iodine in three cases (2, 3 and 9) indicated hyperthyroidism, whereas the clinical diagnosis and other thyroid function tests indicated hypothyroidism for patients 2 and 3 and normal thyroid function for patient 9. These high values could be explained on the basis of a possible ingestion of iodine before the test was performed, although there was no positive evidence of this in the case histories of these patients. If these values are omitted,

References p. 720

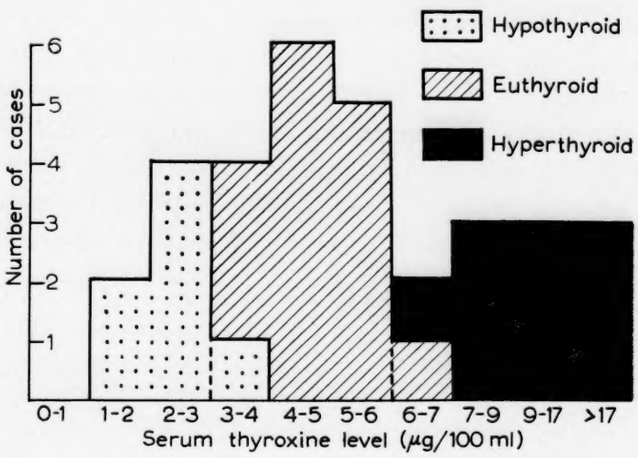

Fig. 2. Distribution of thyroxine levels of serum from patients judged to be hypothyroid, euthyroid and hyperthyroid, on the basis of clinical diagnosis and other thyroid function tests in 32 cases.

the overall mean ratio of thyroxine iodine to protein-bound iodine is 0.58. This ratio calculated from the results obtained in the limited number of analyses carried out shows a tendency to rise with increasing amounts of thyroxine in the serum. The mean ratios for the groups showing hypothyroid, euthyroid and hyperthyroid function were 0.55, 0.58, and 0.79 respectively.

DISCUSSION

Derivative analysis by double isotope dilution is independent of losses occurring during the separation procedure after the addition of labelled carrier. Thus, the general procedure for the estimation of thyroxine in blood using [^{14}C]-labelled acetyl thyroxine (method 1, in Table II) requires the reaction between the serum thyroxine and the [^{3}H]-labelled acetic anhydride to reach completion. Complete reaction in the alternative procedure (method 2) is unnecessary although desirable as the inert thyroxine in the serum and the added [^{131}I]-labelled carrier react with the [^{3}H]-labelled reagent to the same extent and partial reaction finds automatic compensation in the final calculation. The close agreement of the results given in Table II by the alternative methods show that acetylation of thyroxine in blood reaches completion under the experimental conditions used.

The results obtained by method 2 are dependent on the extent of mixing of the labelled compound added and the thyroxine in the serum sample. Mixing of the inert and labelled compounds will only occur if the endogenous thyroxine is present either in the free state or combined by a loose physical linkage to the other constituent compounds. Such a linkage could be hydrogen bonding. Any thyroxine chemically combined is only estimated in so far as the chemical bond is severed by the procedure of acetylation. The close agreement of the results given in Tables II and III indicate that endogenous thyroxine in serum as estimated by the isotope-dilution technique is readily available and not chemically combined by a linkage capable of being severed by a proteolytic enzyme or comparatively drastic alkaline hydrolysis.

The failure to liberate further quantities of thyroxine from serum by hydrolysis and the differences found between the thyroxine iodine and protein-bound iodine values indicate that a considerable proportion of the organic iodine is present in a form other than thyroxine. A number of workers have given estimates of the various iodothyronine levels present in human serum based on the distribution of [^{131}I] between the different compounds at a given time after the administration of the

References p. 720

labelled iodide to the subject. BENUA, DOBYNS AND NIMMER[20] produced evidence that the cases they studied showed that one molecule of 3, 5, 3′-triiodo-thyronine entered the circulation for every three molecules of thyroxine although HYDOVITZ AND ARONS[21] have found it relatively difficult to identify 3, 5, 3′-triiodo-thyronine in the sera of euthyroid subjects (positive in 10% of the cases studied). This result is in contrast to the thyrotoxic cases studied by these authors which were positive in 30% of the subjects. ROCHE *et al.*[22] in their determination of 3, 3′-diiododo-thyronine in

TABLE IV

SERUM THYROXINE LEVELS AND PBI VALUES OF HUMAN BLOOD

Case No.	*Thyroxine (μg/100 ml)*	*Thyroxine iodine (TI) (μg/100 ml)*	*PBI (μg/100 ml)*	*TI/PBI ratio*
A. Hypothyroid				
1	1.8	1.2	3.0	0.40
2	2.0	1.4	13.6	0.10*
3	2.2	1.5	9.7	0.15*
4	2.4	1.6	2.1	0.71
5	2.6	1.7	3.1	0.55
6	2.6	1.7	—	—
7	3.6	2.4	1.7	1.40*
			Mean	0.55
B. Euthyroid				
8	3.0	2.0	1.3	1.54*
9	3.3	2.2	10.0	0.22*
10	3.8	2.2	—	—
11	3.8	2.5	4.6	0.54
12	4.2	2.7	4.4	0.59
13	4.3	2.8	4.8	0.58
14	4.3	2.8	7.8	0.36
15	4.6	3.0	4.1	0.73
16	4.6	3.0	8.7	0.35
17	4.8	3.1	4.5	0.69
18	5.2	3.4	5.6	0.61
19	5.5	3.7	6.4	0.58
20	5.7	3.8	7.2	0.53
21	5.9	3.9	8.7	0.45
22	6.1	4.1	7.7	0.53
			Mean	0.58
C. Hyperthyroid				
23	6.9	4.5	5.6	0.80
24	7.2	4.7	5.3	0.88
25	8.3	5.04	7.2	0.70
26	8.9	6.5	10.5	0.62
27	11.2	7.0	8.2	0.85
28	16.1	10.9	14.5	0.74
29	17.0	—	—	—
30	21.2	—	—	—
31	24.2	15.75	17.5	0.90
32	35.3	23.1	27.6	0.84
			Mean	0.79

* These values were omitted from the calculation of the mean ratio values due to some doubt in the accuracy of the determination of the thyroxine and/or the determination of the protein bound iodine (see text).

References p. 720

human plasma stated that the compound normally accounted for as much as 25% of the serum iodine. All these figures are dependent upon the size of the thyroidal hormone pool and varying sizes of the gland pools of different individual compounds would place doubts upon the quantitative assessment of the proportions of iodothyronines in serum by measurement of [^{131}I] ratios. However, the evidence qualitatively confirms that iodothyronines other than thyroxine are present in serum in comparative amounts.

Although 3, 3′, 5′-triiodothyronine has been identified in rat thyroid[23], no demonstration of its presence in human plasma has been published. However, as pointed out by WERNER AND BLOCK[24] in their recent work showing the presence of the iodotyrosines in normal serum, reliance upon [^{131}I] activity measurement in the detection of such compounds can lead to erroneous conclusions. If there exists a large thyroidal pool of the compound being sought, such excessive dilution may occur of the active compound and the endogenous material in the gland, that the specific activity of the compound thrown out into circulation is so low as to be undetectable. Thus it is possible that 3, 3′, 5′-triiodo-thyronine is present in human blood and has been undetected. However, if this be so, it appears that as the quantities present, in the gland are small, the concentration of this compound in the blood serum is also comparatively small in relation to the thyroxine level.

On the limited evidence given in Fig. 2 it would appear that the degree of activity of the thyroid gland is reflected in the value of the level of thyroxine in the serum. The greater the activity of the gland the higher is the serum thyroxine content. The few anomalous results obtained in the protein-bound iodine values were not reproduced in the thyroxine analyses.

The tendency of the thyroxine-iodine/protein-bound iodine ratios to increase with increasing amounts of thyroxine indicates that this hormone is preferentially secreted into the circulation by the thyroid gland as its activity is raised. Alternatively all the thyroid hormones may be secreted at an equally accelerated rate but the 3, 5, 3′-triiodo-thyronine and the 3, 3′-diiodo-thyronine are removed from the circulation much more rapidly than thyroxine thus raising the proportion of the latter compound remaining in the blood. Some evidence has been published [25–28] to support the second explanation. However, a more complete understanding of the problem must await the determination of the plasma levels of all the thyroid hormones in the blood from subjects of differing degrees of thyroid function.

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SUMMARY

A new method is described for the determination of thyroxine in the blood by double isotope-dilution technique using tritiated acetic anhydride as a radioactive reagent together with [^{131}I]-labelled free thyroxine or [^{14}C]-labelled N-acetyl thyroxine as isotopic carriers. The method appears to estimate the readily available

References p. 720

hormone in serum to an accuracy of 2–3%. The results of analysis of 32 samples of human serum taken from subjects having varying degrees of thyroid activity show that the thyroxine levels obtained follow the general pattern of thyroid activity very closely, in that increased thyroid function produces a high serum-thyroxine level. The ranges of values for hypofunction, euthyroid function, and hyperthyroid function were 0–3.5 μg/100 ml, 3.0–6.5 μg/100 ml, and greater than 6.5 μg/100 ml, respectively. The ratio of the thyroxine-iodine/protein-bound iodine showed a marked tendency to increase with rising thyroid function and the implications of this finding are briefly discussed.

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RENAL EXCRETION OF ELECTROLYTES DURING Tm_{PAH}

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The tubular reabsorption or secretion* of an organic compound can sometimes influence the reabsorption or the secretion of another substance. The numerous conditions which can modify the tubular activity were carefully examined by BEYER[1], who also illustrated the complicated and delicate mechanisms of renal transport concerned.

In the study described here we investigated only a particular aspect of the mutual interference, *i.e.*, the competition between the reabsorption and the secretion activities of the renal tubules.

SELKURT[2] first found a momentary diminution of the ascorbic acid reabsorption during the tubular saturation with *p*-aminohippuric acid (Tm_{PAH}). Several workers[3-5], apart from ourselves, have repeatedly observed that glucose and PAH (*p*-aminohippuric acid) can show a reciprocal depression of their reabsorption and tubular secretion rates when their relative Tm is simultaneously determined. More recently, we have also observed that proteinuria can increase in hypertensive or nephropatic subjects during Tm_{PAH}[6].

In all these cases the transport system governing the reabsorption or tubular secretion of a substance is probably forced to diminish its rhythm of work when another system acting in an opposite direction is engaged in a particularly intense transport activity.

On the other hand, the infusion of an hypertonic solution of sodium or potassium chloride reduces the ascorbic acid reabsorption[2] and increases the PAH clearance[7], whereas no diminution of the glomerular filtration rate and renal plasma flow occurs. Possibly the hypertonic saline solution acts in a complex way on the tubular cells, so that it seems questionable to speak strictly of an interference of the electrolytes on the tubular transport mechanisms.

Thus it appears interesting also to examine the reverse aspect of the matter, *i.e.*, if the reabsorption of water and electrolytes can be influenced by the saturation of the excretory capacity of the proximal tubules with a given substance. STAMLER[8] thought that this competition was possible, but later considerations forced him to change his opinion radically[9]. The problem, as far as we know, has not been debated more recently, and we believe our results in this field to be of interest.

MATERIAL AND METHODS

The experiments were carried out on a group of 25 patients, 12 of whom had renal disease of various origin (acute or chronic glomerulonephritis, nephrosis, renal stasis), and 13 arteriosclerosis or essential hypertension.

* In this report the terms "excretion" and "secretion" will be employed indifferently, since it is not possible to distinguish, as things stand, between the simple transfer of substance from the interstitial fluid to the tubular lumen (excretion) and the more complex operation of secretion that requires, on the contrary, a modification in the chemical constitution of the substance itself.

References p. 727

In the days preceding the test, the majority of the patients were allowed a free diet; only the acute nephritic patients were subjected to a restriction of the sodium chloride intake. Early in the morning, sodium thiosulphate (4%) and sodium *p*-aminohippurate (PAH Simes: 0.4%) were rapidly infused (8–10 ml/min) intravenously; after 5 min the infusion speed was reduced to 4 ml/min and maintained constant. After allowing 25–30 min for equilibration, the bladder was thoroughly washed with water, and the urine collected by catheter over two 15–30 min periods.

At the beginning of each clearance period, venous blood samples were taken from the arm opposite that into which the infusion had been made, and transferred into heparinized tubes. Then a new solution of sodium thiosulphate (3%) and sodium *p*-aminohippurate (8–9%), at the speed of 5–7 ml/min, was infused. After allowing about 30 min for equilibration, two new clearance periods (15–20 min) were examined.

The following determinations were carried out in duplicate on blood and urine:

a) thiosulphate, by BRUN's method [10];

b) *p*-aminohippurate, by the method of SMITH *et al.*[11];

c) sodium and potassium, by flame spectrophotometry (Beckman DU);

d) chloride: plasma Cl, by the method of SCHALES AND SCHALES[12]; urinary Cl, by the method of VOLHARD-HARVEY[13], modified by applying the calculations necessary to correct the total values of chloruria from the interference of the urinary thiosulphate.

Employing the usual formulae, we calculated the glomerular filtration rate (GFR: thiosulphate clearance), renal plasma flow (RPF: PAH clearance), tubular saturation limit for PAH (Tm_{PAH}), clearances of electrolytes (Na, K, Cl) and their percentage elimination with respect to the filtered amount. All these data were corrected to a standard surface area of 1.73 sq.m.

In 10 of these patients (7 hypertensive and nephropatic) the plasma and urine $\triangle$ and the osmotic clearance have been determined with Beckman's osmometer.

RESULTS AND DISCUSSION

When the data for GFR and RPF* are considered, it is possible to subdivide the 25 patients into three groups. The *first* (*A*), of 8 subjects (7 hypertensive and 1 nephropatic), had normal renal tests, the *second* (*B*), of 6 subjects (2 hypertensive and 4 nephropatic), had renal tests partially altered**, and the *third* (*C*), of eleven subjects (four hypertensive and seven nephropatic), had markedly altered tests (Table I).

In the basal state, that is during the first infusion of sodium thiosulphate and PAH, the urine was almost always hypertonic, as shown by the presence of a deficit of excreted water in relation to the osmolar clearance ($T^c_{H_2O}$); this may be due to the amount of thiosulphate contributing to the urinary hypertonicity[15].

When sodium PAH was infused so rapidly as to overcome the saturation limit (Tm), clear and unequivocal changes in the composition of urine were observed.

A. In subjects functionally normal

The GFR remained constant, while the rate of the *urinary flow* increased considerably (average + 150%). The plasma *sodium* concentration remained almost constant.

* According to CORÀ[14] the values considered as normal are: 131.3 ± 11.2 for GFR, 633 ± 67.3 for RPF (ml/min), and 77.6 ± 10.3 for Tm_{PAH} (mg/min).

** The mean values for renal clearance tests, as reported in Table I, are normal; however, the individual values from which such averages were calculated were significantly abnormal.

References p. 727

TABLE I

MEAN VALUES OF FILTRATION RATE AND URINARY EXCRETION OF WATER ,SODIUM, CHLORIDE AND POTASSIUM BEFORE AND DURING Tm_{PAH}

	GFR	*RPF*	Tm_{PAH}	*Water*			*Sodium*			*Chloride*		*Potassium*	
	ml/min	*ml/min*	*mg/min*	*ml/min*			*mequiv./min*			*mequiv./min*		*mequiv./min*	
				load	*filter*	*elimin.*	*load*	*filter*	*elimin.*	*filter*	*elimin.*	*filter*	*elimin.*
Group A	130			5	130	2.15	2.6	18.4	0.440	14.95	0.274	0.585	0.107
(8 subj.)	130	555	83	7	130	5.44	5.0	18.6	1.073	14.95	0.557	0.559	0.169
						(+153%)			(+144%)		(+103%)		(+58%)
Group B	132			5	132	2.46	2.6	19.0	0.464	14.95	0.349	0.634	0.095
(6 subj.)	132	573	75	7	132	4.80	5.0	19.0	0.916	15.00	0.582	0.634	0.181
						(+95%)			(+97%)		(+67%)		(+90%)
Group C	65			5	65	2.57	2.6	9.4	0.461	7.29	0.275	0.305	0.084
(11 subj.)	65	322	46	7	65	4.66	5.0	9.6	0.857	7.29	0.411	0.299	0.117
						(+81%)			(+86%)		(+50%)		(+39%)

The 25 subjects are divided into Groups A: subjects with normal renal function, B: subjects with slightly altered renal function tests, and C: subjects with markedly alterated renal function tests.

The urinary sodium concentration was often reduced; however, owing to the simultaneous increase of urinary flow, increased sodium elimination per time unit (a little less than +150%, in respect to the basal state) was observed, which resulted in an increase of the clearance values. The tubular reabsorption of the electrolyte, as a percentage was consequently smaller during Tm_{PAH}.

Chloride. The changes of the chloride excretion during Tm_{PAH} were similar, although less accentuated. The plasma concentration tended to remain constant with small variations; the elimination of chloride per time unit was generally greater (as an average doubled) in comparison with the initial values. Also the clearance values were always higher, and the percent tubular reabsorption of chloride was lower.

Potassium. The plasma concentration of potassium often tended to diminish during the tubular saturation with PAH, and, almost always, the urinary excretion rate per time unit increased, although less than with sodium and chloride (an average increase of 50% in respect to the basal values). Consequently the clearance values became higher.

Water. Its percentile elimination with respect to the filtered amount increased during Tm_{PAH}; its increase was of the same order of magnitude as for sodium (+153%). Also the osmolarity of the urine appeared reduced during Tm_{PAH}. This indicates that the water elimination was proportionally greater with respect to the elimination of solutes than before the saturation of the tubular system. The urine, though hypertonic, was relatively more dilute than before (the ratio U_{osm}/P_{osm} was smaller).

B. In subjects with renal functions moderately altered

The results were similar to those obtained in the patients belonging to the group *C*.

C. In subjects with markedly abnormal renal functions

In these subjects the GFR again remained constant and a considerable increase of the *urine volume* was observed, although this was smaller than in the subjects considered normal. The plasma *sodium* concentration showed moderate variations.

References p. 727

Although the GFR of these patients was about 50% of the normal values, the urinary sodium concentration was of the same degree as that of the first group (Table II). The sodium elimination per time unit increased during Tm_{PAH}, although not as much as in the normals (+86% against +144%).

TABLE II

COMPOSITION OF URINE EXCRETED BEFORE AND DURING Tm_{PAH}
(mequiv./l)

	Group A		*Group B*		*Group C*	
	before	*during*	*before*	*during*	*before*	*during*
Na^+	204	197	189	190	180	184
K^+	39	31	39	38	33	25
Cl^-	127	102	142	121	107	88
Thiosulphate	30	16	24	16	17	13
p-Aminohippurate	24	105	18	99	18	76
Cations	243	228	228	228	213	209
Anions	181	223	184	236	142	177

The *chloride* elimination behaved in a similar way, the only difference being that the percentile elimination of the filtered electrolyte increased less during Tm_{PAH}. *Potassium* also tended to maintain its urinary concentration within the range found in subjects considered normal, although GFR was reduced to about half the original value.

The elimination of *water* during Tm_{PAH} in the subjects with altered renal function increased by 80%, *i.e.* only half the value obtained with subjects of the first group.

When discussing the elimination of water and sodium, it must be kept in mind that in the basal state as well as during Tm_{PAH}, solutions of sodium thiosulphate and sodium *p*-aminohippurate were infused. In the normal subjects, 43% of the water introduced was excreted before Tm_{PAH} and 78% during Tm_{PAH}; in the subjects with altered renal function ,the percentile increase during the tubular saturation was much less. As regards sodium, in normal subjects the elimination rate with respect to the administered load was 16.9% in the basal state and 21.5% during Tm_{PAH}, while in subjects with impaired renal functions the value remained constant. The sodium load (in the form of salts of thiosulphuric and *p*-aminohippuric acids) did not seem to exert any influence on the plasma concentration of Na, which remained practically constant during Tm_{PAH}, in spite of the fact that the infused amount of sodium was twice the amount infused during the basal periods.

DISCUSSION AND CONCLUSIONS

The results indicate that the tubular reabsorption of the filtered sodium, potassium and chloride is reduced when the tubular system is saturated by an intravenous load of *p*-aminohippurate. In other words, the proportion of filtered electrolytes eliminated with urine is increased during Tm_{PAH}. This seems to occur both in subjects with normal renal function and, to a smaller extent, in subjects with altered renal function.

This increase in the elimination of electrolytes is directly proportional to the percentile elimination of the filtered water (V/GFR · 100); this relationship, strikingly conspicuous in the case of sodium, is very clearly shown in Fig. 1, where the values of

References p. 727

water and sodium excretion are expressed as percentages of the filtered amounts (see also Table I).

The increase of C_{osm} during Tm_{PAH} is related to the rise in diuresis (Fig. 2), but is comparatively smaller, and the urine, though remaining hypertonic, is more dilute with respect to basal conditions.

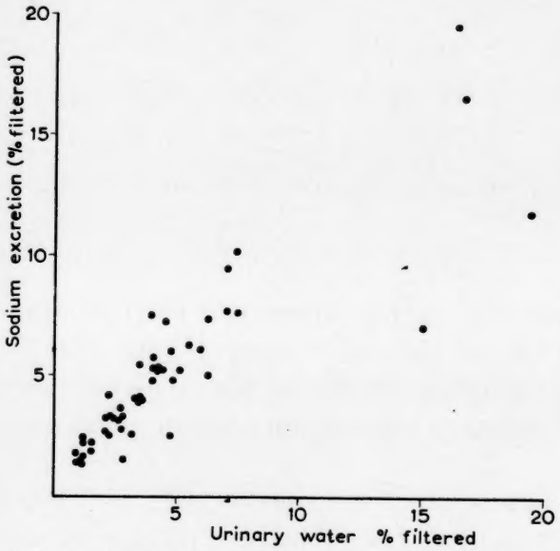

Fig. 1. Correlation between sodium excretion (% filtered) and urinary water, before and during Tm_{PAH}.

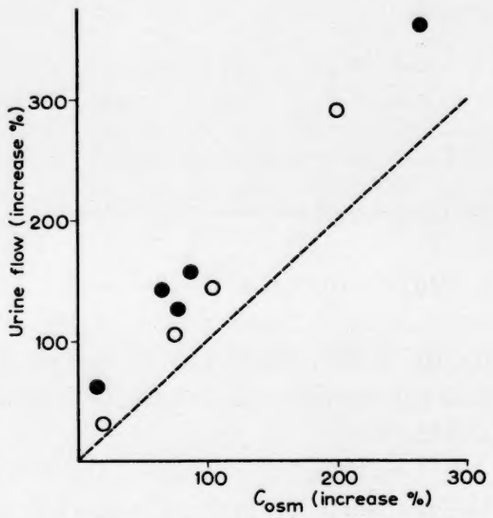

Fig. 2. The percentile increase of urinary flow during Tm_{PAH} is always greater than that of C_{osm}, both in normals (●) and in subjects with altered renal functions (○).

Thus the kidneys seem to face a PAH load by a slight reduction in the urinary concentration of sodium, chloride and potassium, and by a considerable increase in the water elimination; more electrolyte is excreted in a still greater water volume. Although the water excretion is markedly greater than the basal values, it is still insufficient to make the urine isosmotic with the accompanying plasma. This was observed both in the normal subjects and in those with abnormal renal function; the latter, in spite of a considerably reduced GFR, maintained the elimination of electrolytes and water within the inferior normal range both before and during Tm_{PAH}. This was made possible by the reduced tubular reabsorption.

It is known that the sodium reabsorption takes place mainly as a transference together with a corresponding number of fixed anions (mostly chloride), but partly as an exchange with H^+ and K^+[16]. It is easy to analyze the behaviour of the sodium filtered before and during Tm_{PAH} (Table III); it appears that the amount of sodium reabsorbed along with chloride ions and through the exchange with H^+ decreases, while the amount exchanged with K^+ increases. The interpretation of these changes occurring during Tm_{PAH}, and involving all the electrolytes, seems obscure.

Concerning sodium, the observation of STAMLER *et al.*[8], who first ascribed the increased sodium elimination during Tm_{PAH} to a competitive inhibition of the tubular reabsorption, should be recalled. In a later report, however, the same author[9] postulated that the natriuretic effect is due both to the sodium load infused with PAH, and to the necessity of PAH fixing cations during its excretion.

This hypothesis is worth consideration and, to some extent, our results agree with

References p. 727

TABLE III

TUBULAR REABSORPTION OF SODIUM BEFORE AND DURING Tm_{PAH}

Reabsorption of sodium	*Normal values*	*Group A*			*Group B*			*Group C*		
		before	*during*	*difference*	*before*	*during*	*difference*	*before*	*during*	*difference*
Bound to fixed anions (mostly Cl^-)	12.000	14.676	14.393	—0.283	14.601	14.418	—0.183	7.015	6.879	—0.136
Exchange Na^+ — K^+ (K^+ excreted)	0.050	0.107	0.169	+0.062	0.095	0.181	+0.086	0.084	0.117	+0.033
Exchange Na^+ — H^+ ($NaHCO_3$ reabs. etc.)*	3.250	3.177	2.965	—0.212	3.840	3.489	—0.351	1.840	1.747	—0.093
Total	15.300	17.960	17.527	—0.433	18.536	18.088	—0.446	8.939	8.743	—0.196

The values are expressed in mequiv./min. The normal values are those reported by G. H. MUDGE, (*Am J. Med.*, 20 (1956) 448.

* Non-experimental calculated values.

it. During maximal secretion of PAH the increases in the sodium and PAH elimination are fairly similar (+0.491 mequiv./min for the Na and +0.419 mequiv./min for for the PAH: Table IV). However, it must be pointed out that in all our patients increased elimination during Tm_{PAH} involved not only sodium but also an anion such as chloride.

The hypothesis that at the moment of saturation of the tubular cells with PAH the reabsorption process is hindered, furnishes a more comprehensive interpretation. LEVY AND ANKENEY[17] found a depressive influence of sodium loads on the tubular transport of PAH, bicarbonate, sulphate, glucose and ascorbic acid. Under our experimental conditions, we have found that during Tm_{PAH} the tubular function, as regards the transport of the electrolytes, appeared depressed, although the effect was variable.

TABLE IV

MEAN VALUES OF EXCRETION OF ELECTROLYTES

in mequiv./min in 25 subjects before and during Tm_{PAH}

	Na^+	*K^+*	*Cl^-*	*Thios$^-$*	PAH$^-$	*Cations*	*Anions*
Before Tm_{PAH}	0.456	0.095	0.299	0.056	0.048	0.551	0.403
During Tm_{PAH}	0.947	0.158	0.517	0.075	0.467	1.105	1.059
Difference	0.491	0.063	0.218	0.019	0.419	0.554	0.656

In our opinion the inhibiting action of PAH on the tubular transport of many different urinary components (electrolytes, proteins, ascorbic acid, glucose) may be caused by its interference with the intracellular enzymic and/or energetic mechanisms*. In this connection, the observation of CROSS AND TAGGART[18] that the PAH accumulation in kidney slices requires the integrity of the oxidative phosphorylation mechanisms may be of interest.

* Similar interference with intracellular enzymic or energetic mechanisms has been postulated by SELKURT[2] for the competition between PAH secretion and ascorbic acid reabsorption, by LEVY AND ANKENEY[17] for the competition between Na load and PAH secretion, and by JOSEPHSON *et al.*[19] for the competition between PAH and diodrast secretion.

References p. 727

Thus it appears likely that the tubular cells which are forced to secrete at the maximal speed the PAH infused intravenously during the *Tm* determination, utilize a part of the energy normally reserved for the reabsorption of glucose, proteins, electolytes and ascorbic acid, so that the reabsorption of these is partly compromised.

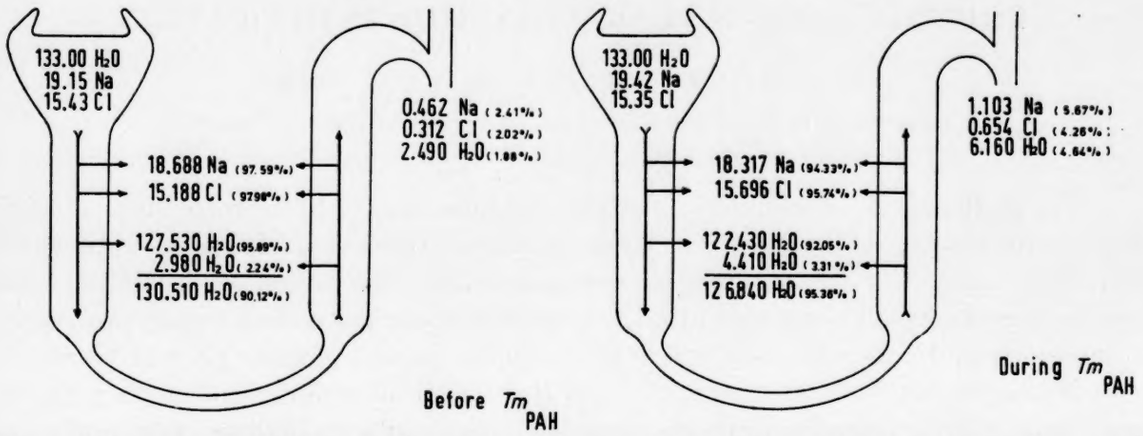

Fig. 3. Diagrams of filtration, reabsorption and excretion (average rate) of water, sodium and chloride in 5 subjects with normal renal functions (group A).

SUMMARY

In 25 hypertensive and nephropatic subjects, the water, sodium, chloride and potassium excretion during the saturation of the proximal tubules with *p*-aminohippuric acid (Tm_{PAH}) has been studied. The significant diminution of the tubular reabsorption of water and electrolytes, which was found in all the patients, is thoroughly discussed.

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PROTECTIVE EFFECT OF ORNITHINE AND ASPARTIC ACID IN CHRONIC CARBON TETRACHLORIDE INTOXICATION

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The inclusion of L-arginine[1] in a mixture of nine essential L-aminoacids strikingly reduces the toxicity of high concentrations of this mixture in rats. It has been shown[2–5] that the toxic effect of ammonia is counteracted by this amino acid. GREENSTEIN and his coworkers[6] demonstrated that the combined administration of non-protective compounds and sub-protective levels of L-arginine have a similar protective effect.

NAJARIAN AND HARPER[7] investigated the action of arginine in human patients with various liver diseases (cirrhosis, jaundice, etc.) and with high levels of ammonia in the blood. Arginine injected intravenously led to a striking fall in the level of ammonia nitrogen and to an increase of blood urea. Similar results were recently obtained by FAHEY[8], who confirmed the antitoxic action of L-arginine in human subjects injected with amino acid mixtures.

These and other reports emphasize the essential role of L-arginine in the administration of such mixtures. It has been assumed that the protective effect of L-arginine is due in part to an acceleration of the hepatic KREBS-HENSELEIT[9] urea cycle, which results in an increased rate of disappearance of toxic ammonia.

It was considered desirable to ascertain if other substances related to urea biosynthesis could produce an effect similar to that of arginine. We have therefore tested the action of two amino acids (ornithine and aspartic acid), which are considered the best catalysts for urea biosynthesis[10–11], on livers chronically injured with carbon tetrachloride. The tetrachloride causes an experimental cirrhosis in rats like the Morgagni-Laënnec cirrhosis of human pathology[12]. The injury is reflected by a remarkable damage to the hepatic cell with disturbances of the liver functions (principally the urea synthesis), as well as of other general functions in the organism.

In the first series of experiments the weight increase of young rats during growth was determined, and in a second series of experiments the liver lipids and glycogen content of adult rats were determined.

EXPERIMENTAL

Series I

Albino rats weighing 75–85 g were supplied from the Morini breeding colony of Reggio Emilia. The animals were fed the Coward diet (plus Vit. A and D) and lived in individual cages at suitable temperatures (about 20–25°). They were divided into 4 groups. The first group was used as control (fed on normal Coward diet only). The rats of the second group were treated with CCl_4 as indicated below. Those of the third group were subjected to CCl_4 intoxication, and were fed a normal diet supplemented by ornithine and L-aspartic acid. The rats of the fourth group were fed the Coward diet plus ornithine and aspartic acid, without the CCl_4 treatment. The amino

References p. 732

acids (Hoffman–La Roche) administered *per rat* were used in amounts of 2.5 mmole/kg of weight per day. They were dissolved in phosphate buffer $M/15$ (final pH = 7.4), and administered by stomach tube twice a day (with an interval of 8 h). The rats of the first and second group were treated analogously with the buffered solution only.

The poisoning by CCl_4 was done according to the following procedure. The air in a closed glass container of 22.5-l capacity was saturated with CCl_4. Four rats (one of each group) were simultaneously placed in the glass container for 7 min daily for 23 consecutive days. The animals were weighed daily to observe the extent of growth.

Series II

Male Morini rats weighing 190–210 g were employed in these experiments. They were divided into four groups and treated under the same conditions as described above. The treatments were maintained for 50 days, after which the rats were decapitated.

The livers were immediately removed and dehydrated by several washings with 95% ethanol. The following procedures were carried out on weighed pieces of liver.

1) *Estimation of total lipids*[13]. The lipids were extracted with boiling absolute ethanol in the Kumagawa apparatus (8 h). The extract was evaporated to dryness under a continous flow of nitrogen at reduced pressure. The residue was eluted with ether and filtered through Schleicher-Schull No. 5892 papers. The ether filtrate was evaporated carefully to dryness under vacuum and the lipids were estimated by weighing the residues.

2) *Estimation of glycogen.* The phenol–sulfuric acid colorimetric procedure according to MONTGOMERY[14] was used.

The numerical data reported below represent the average values of duplicate analyses performed on the separate livers of rats treated in the same way.

RESULTS

The average weight increase of rats in grams during the experimental period is shown in Fig. 1. The data for the fourth group are not reproduced since they are similar to those of the first group. Column B refers to weight increase during the treatment (23 days) and column A to that observed during 50 days (*i.e.* including a period before and after the treatment).

TABLE I

TOTAL LIPID CONTENT IN RAT LIVER
Average values in g% of wet tissue

Treatment (50 days)	*Total lipids*	*Differences**	*Differences***
a) None	6.27	—	+1.41
b) Chronic poisoning by CCl_4	4.86	—1.41	—
c) As b) + ornithine (50 mg/rat/day) + aspartic acid (32 mg/rat/day)	6.21	—0.02	+1.35
d) Ornithine (50 mg/rat/day) + aspartic acid (32 mg/rat/day)	6.41	+0.14	+1.55

* Differences as compared with control rats (a).
** Differences as compared with CCl_4-poisoned rats (b).

References p. 732

In both columns the average increase of weight is much lower in poisoned rats than in control animals. In the same period the rats treated with carbon tetrachloride plus ornithine and aspartic acid showed weight increases equal to those of the control rats.

The result summarized in Table I show the lipid contents of the liver in rats from the second series of experiments.

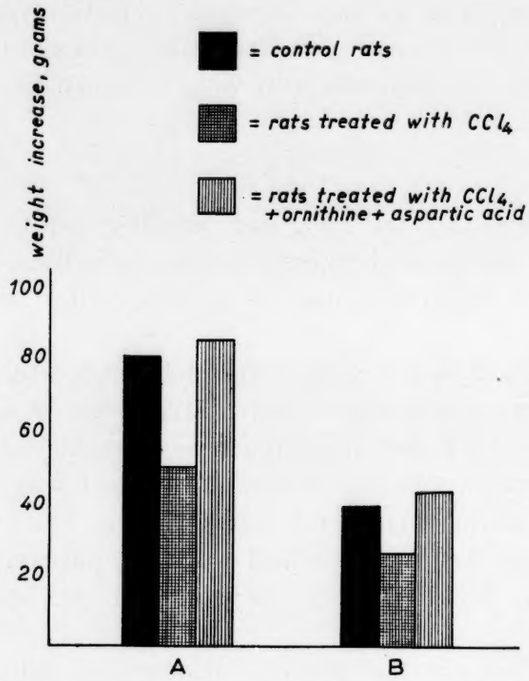

Fig. 1. Weight increase of normal rats, poisoned rats, and protected rats; A = weight increase during the experimental period (50 days). B = weight increase during the treatment (23 days).

The data in Table I indicate a striking decrease of liver fats in the rats treated with carbon tetrachloride whereas the levels are close to normal in protected rats.

In earlier papers[15-16] the effects of administering ornithine, aspartic acid or arginine singly, and of an arginine–aspartic acid mixture were described. The results indicate in every case that the protective efficiency of these treatments is not so high as that of the ornithine–aspartic acid mixture.

The level of hepatic glycogen shows a similar behaviour in poisoned and protected rats. Table II shows the glycogen content of the livers.

TABLE II

GLYCOGEN LEVEL IN RAT LIVER

Average values in g % of wet tissue

Treatment (50 days)	*Glycogen*	*Differences**	*Differences***
a) None	4.51	—	+2.86
b) Chronic poisoning by CCl_4	1.65	—2.86	—
c) As b) + ornithine (50 mg/rat/day) + aspartic acid (32 mg/rat/day)	2.57	—1.94	+0.92
d) Ornithine (50 mg/rat/day) + aspartic acid (32 mg/rat/day)	4.62	+0.11	+2.97

* Differences as compared with control rats (a).

** Differences as compared with CCl_4 poisoned rats (b).

References p. 732

The protective efficiency, as evaluated from the liver glycogen levels, is less marked than that evaluated on the basis of lipid content. This may be partly due to some variation in hepatic glycogen which depends on uncontrolled environmental and nutritional factors.

These results and those previously published[15, 16] strongly suggest that the ornithine–aspartic acid mixture gives the best results in protecting rats from carbon tetrachloride intoxication.

Fig. 2 shows a comparison of the protective efficiency obtained by the various treatments on the basis of the total fat content of the liver. This efficiency is expressed as per cent of the normal lipid content. The ornithine–aspartic acid mixture appears to exhibit the best protective activity (about 96%).

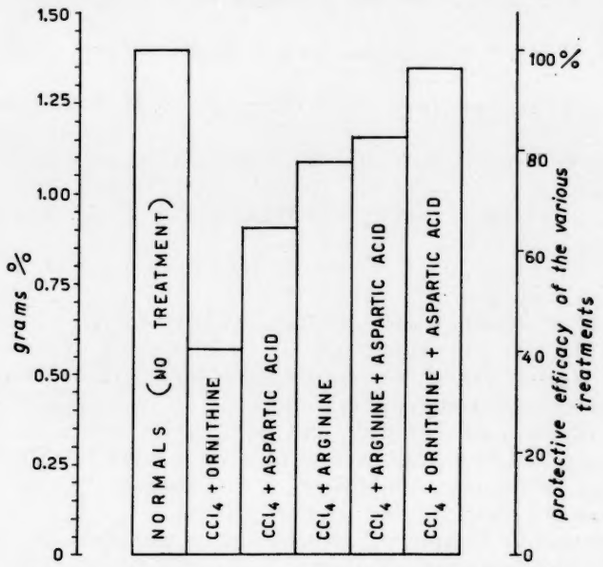

Fig. 2. % increase of total liver fats (on left).
% protective efficiency of various treatments (on right).

Our studies suggest that the mode of action of these amino acids (aspartic acid, ornithine) is specific and that they could be regarded as "catalysts" for urea biosynthesis.

The ammonia and other catabolic nitrogen-containing substances which are continuously produced in injured liver, may be removed by the administration of amino acids that catalyze the hepatic urea cycle.

WALSHE[17] found that glutamic acid had a protective action in hepatic diseases, and other authors [1–8] have described the similar action of arginine. Our studies suggest that two other amino acids (ornithine and aspartic acid) may play a significant role as antitoxic agents in chronic liver diseases.

ACKNOWLEDGEMENTS

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References p. 732

SUMMARY

The effects of two amino acids (ornithine, aspartic acid) in chronic poisoning by CCl_4 in rats have been investigated. The protective efficiency obtained has been evaluated on the basis of weight increase and of the total fats and glycogen contents of the liver. The efficiency reaches 96% when an ornithine–aspartic acid mixture is used. The results obtained are presumably due to a specific action of the amino acids, *i.e.* they act as "catalysts" in urea biosynthesis. The action consists, at least in part, in an acceleration of the classic KREBS–HENSELEIT urea synthesis mechanism in the liver. The use of ornithine–aspartic acid mixture is suggested in the treatment of liver diseases.

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VERBESSERUNGEN ZUR METHODE DER EIWEISSBESTIMMUNG MITTELS Cu(II) UND PHENOLREAGENS NACH FOLIN-CIOCALTEU

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In einer früheren Publikation[1] stellten wir vergleichsweise verschiedene Methoden der Liquoreiweissbestimmung einander gegenüber. Wir gelangten dabei zum Schluss, dass die kurz mit "Folin–Cu-Methode" bezeichnete und anderweitig im Detail beschriebene[2] Ausführung sich als sehr geeignet erweist für klinische Routineuntersuchungen und im Besonderen, wo es sich um die Bestimmung kleinster Flüssigkeitsmengen handelt. Auf gelegentlich auftretende Fehlermöglichkeiten und die Notwendigkeit ihrer Eliminierung wurde damals schon hingewiesen.

Nach anderthalbjähriger Anwendung dieser Methode überblicken wir heute nicht nur mehrere Hunderte von Proben, welche vergleichsweise meist auch mit der bisher verwendeten Sulfosalicylsäuretrübung gemessen wurden, sondern wir haben auch reiche Erfahrungen in bezug auf die erwähnten Fehlermöglichkeiten und vor allem deren Vermeidung sammeln können. Von diesen letzteren soll in der vorliegenden Mitteilung die Rede sein.

A. BLINDWERT

Ein normaler Blindwert erscheint als wasserklare, ganz leicht blaustichige Flüssigkeit. Gegen Wasser gemessen zeigt er im Messbereich um 755 mμ eine Extinktion von durchschnittlich etwa 0.050 $\pm$ 0.020; Werte über 0.080 werden auch von Auge schon sofort als dunkler erkannt. Grosse Mühe bereitete uns eine Zeit lang das vereinzelte und gänzlich willkürliche Auftreten von solchen, dunkler blau gefärbten Blindwerten, welche das Resultat verfälschen.

Als Störfaktoren, die sich natürlich im Teströhrchen so gut wie im Blindröhrchen auswirken können, aber nur in letzterem eindeutig zu erkennen sind, kamen einerseits die im gleichen Laboratoriumsraum verwendeten Spraymittel für die Chromatographie, anderseits eventuelle Unreinheiten an den Glaswaren in Frage. Wegen der hohen Empfindlichkeit des Tests (4 μg pro Testvolumen von 0.1 ml bewirken ja bereits eine Wertveränderung von 0.020 *E*!) wurden Glaswaren und Pipetten ausgesondert, welche inskünftig streng separat gehalten, nur für diesen Test verwendet und jeweils speziell gereinigt wurden. Ausserdem verlegten wir die Bestimmung vorübergehend in einen anderen Raum. Trotzdem trat in den an verschiedenen Tagen und mit verschieden frischen Reagenzien angesetzten Serien von mehreren Blindwerten immer noch, aber wie bisher immer nur ganz vereinzelt einmal ein blauer Blindwert auf.

Die Zugabe verschiedener Putzmittel wie Seife, Per, Kaliumbichromat, Säuren, Laugen, organische Lösungsmittel etc. (in als Verunreinigung möglichen Spuren natürlich), wie auch verschiedene Salzkonzentrationen (0.7–2.8% NaCl) waren ohne

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Literatur S. 740

jeglichen Einfluss auf die Höhe des Blind- wie des Testwertes. Dasselbe gilt übrigens für die bei der direkten Eiweissbestimmung im UV-Licht so störende Ascorbinsäure (1–10 mg% unmittelbar vor Bestimmung zu Liquores oder Reineiweiss-Lösungen zugegeben). Von verschiedenen Chromatographier- und Spraymitteln erwiesen sich bei künstlicher Zugabe einzig *p*-Dimethylaminobenzaldehyd und in geringerem Ausmass Ninhydrin als gefährlich, dies aber in Konzentrationen, die kaum auf dem Luftweg ins Reagenzglas gelangen können. Ausserdem hätte dies in keiner Weise zu erklären vermocht, wieso beispielsweise unter 7 nebeneinander angesetzten, identischen Blindproben 6 normal und gerade nur eine mitten heraus blau waren.

Der Fehler musste also offensichtlich woanders liegen und er konnte auch tatsächlich in einem sehr banalen Umstand entdeckt werden.

Wenn hier trotzdem kurz über dessen Aufklärung berichtet wird, so deshalb, weil es sich um eine Reihe von Handgriffen handelt, die routinemässig, z.T. unbewusst, ausgeführt werden. Wir halten es aber nicht für nötig, dass jeder, der sich in dieser Methodik neu einüben will, zuerst selbst über diese Schwierigkeiten stolpern muss; zumal bei deren Nichtbeachtung die an sich gute Methode in Misskredit geraten müsste.

Wie für manche andere Untersuchung in unserem Laboratorium wurden auch für diese Farbreaktion kurze Reagenzrohre verwendet. Bei diesen kann es nun vorkommen, dass bei heftigem Schütteln ein winziges, kaum bemerkbares Flüssigkeitströpfchen herausspritzt, an der meist in der Nähe der Öffnung befindlichen Hand des Untersuchers abprallt und wieder ins Röhrchen zurückfällt. Eine solche Berührung mit Haut, auch wenn diese noch so trocken scheint oder frisch gewaschen wurde, genügt in den meisten Fällen, um eiweisshaltigen Schweiss ungewollt in die Lösung zu bringen und den Blindwert damit u.U. bis zu 0.100 E zu verfälschen (wie gesagt: 4 μg = 0.020 E).

Nach Abklärung dieser Sachlage war es möglich, Mal für Mal wissentlich "falsche Blindwerte" zu reproduzieren, resp. deren bis dahin "mysteriöses" Auftreten zu vermeiden, wenn dafür gesorgt wurde, dass der beschriebene Kontakt mit Haut unmöglich war. Seither wird nur noch mit *langen Reagenzrohren* gearbeitet und diese werden so gehalten, dass das Zurückprallen eines ev. doch noch entweichenden Tröpfchens ausgeschlossen ist. Im gleichen Sinne ist natürlich auch der *Rand* des Reagenzrohres, über welchen die Versuchsflüssigkeit in die Messküvette ausgegossen wird, nicht mit den Fingern zu berühren; dasselbe gilt für Pipettenspitzen usw. Überdies setzen wir bei jeder grösseren Messreihe 2 Blindproben an und überzeugen uns durch Messung gegen Wasser von deren Übereinstimmung und absoluter Höhe; Werte über E = 0.080 werden als unzulässig eliminiert. Wir sichern uns auf diese Weise vor dem Verlust ganzer Messreihen im Falle, dass aus irgendwelcher Unachtsamkeit doch einmal ein blauer Blindwert auftreten sollte, indem wir dann ganz einfach auf das andere, in Ordnung befundene Röhrchen zur Verwendung als Blindwert zurückgreifen können.

Unter diesen Voraussetzungen hat sich die Folin–Cu-Methode als zuverlässig und präzis erwiesen.

B. TESTWERT

Es ist zu bedenken, dass alle im vorherigen Kapitel erwähnten Fehler natürlich auch im Teströhrchen unterlaufen können, mit dem Unterschied aber, dass sie hier nicht augenfällig zutage treten. Umsomehr ist sauberstes Arbeiten Bedingung.

Literatur S. 740

Kommt es auf höchste Präzision an, so empfiehlt es sich, die zu messende Lösung gerade im Doppel anzusetzen, was bei der geringen Flüssigkeitsmenge und dem unbedeutenden Arbeitsaufwand nicht ins Gewicht fällt. An hunderten solcher Doppelproben hat sich die früher gemachte Angabe bestätigen lassen, dass der normale Streubereich ($M \pm 2\sigma$) identischer Lösungen nicht mehr als $\pm$ 0.020 E entsprechend $\pm$ 4 mg% beträgt.

Lösungen, welche eine Extinktion über 0.6 ergeben, sind zu verdünnen und nochmals zu bestimmen. Wie Fig. 1 zeigt, besteht im Bereich zwischen 0–150 mg% Eiweiss (E = 0–0.65) mehr oder weniger lineare Abhängigkeit, indem die Extinktionswerte auf einer nur ganz leicht nach oben durchgebogenen Geraden liegen. Darüber findet ein rasches Umbiegen der Kurve statt; das Reagens ist erschöpft.

Bei stark verdünnten Lösungen (E unter 0.10) wird der methodische Fehler im Verhältnis zur abgelesenen Extinktion gross. Es ist darum vorteilhaft, die Reaktion mit 0.2 ml anstatt 0.1 ml durchzuführen. An den Volumina der übrigen Reaktionsteilnehmer ist nichts zu verändern. Handelt es sich um Untersuchungen, bei denen mehrheitlich hochverdünnte Lösungen bestimmt werden sollen, so legt man sich selbstverständlich eine entsprechende Eichkurve für 0.2 ml Versuchsflüssigkeit an. Treten solche niedrige Konzentrationen aber nur vereinzelt in Serien von höheren Werten auf, so kann man sich ebensogut auf die folgende Weise behelfen:

TABELLE I

VERGLEICH DER EIWEISSBESTIMMUNG MIT 0.1 ml UND MIT 0.2 ml TESTFLÜSSIGKEIT NACH DER IM TEXT (B) BESCHRIEBENEN UMRECHNUNG

(Mod. Methode, vgl. D und E)

C.S.F. Nr.	*mg% Eiweiss*		*Diff.*	*C.S.F. Nr.**	*mg% Eiweiss*		*Diff.*
	0.1 ml	*0.2 ml*			*0.1 ml*	*0.2 ml*	
170	24	25	+1	170	22.5	24	+1.5
171	35	36.5	+1.5	171	36	36	0
171	37	41.5	+4.5	183	35.5	34.5	—1
172	40	40	0	172	43.5	40	—3.5
173	35	35	0	173	36	34.5	—1.5
174	41	40.5	—0.5	174	44	40	—4
175	31	29	—2	175	30	30	0
176	26.5	28	+1.5	176	28	26.5	—1.5
177	68	68.5	+0.5	177	69	72	+3
178	68	70	+2	178	69	70.5	+1.5
184	35.5	35	—0.5	186	30.5	31	+0.5
185	32.5	31.5	—1	185	30	30	0
187	34	32.5	—1.5	187	36	34	—2
188	33.5	33	—0.5	188	37	36	—1
189	22	23	+1	189	25.5	26.5	+1
190	45.5	47.5	+2	191	46.5	47	+0.5
192	50	50.5	+0.5	195	46.5	47.5	+1
196	27	24.5	—2.5	197	46.5	45	—1.5
198	28	26	—2	199	43	43	0
200	36	36	0	201	62	60.5	—1.5
202	45	43	—2	203	37.5	36.5	—1
204	57.5	57.5	0				
Summe Diff. (n = 22)			+2	(n = 21)			—9.5
			—9.5				
Total: (durchschn. Diff.)			—7.5 : 43 = 0.17 ± 1.66 mg%				

* In allen Fällen, wo eine Liquor-Nummer mehrfach vorkommt, wurde die Vergleichsbestimmung von einer anderen Laborantin zur Doppelkontrolle wiederholt.

Literatur S. 740

Man bestimmt die im vorherigen Versuch zu niedrig herausgekommene Lösung nochmals mit jetzt 0.2 ml Testflüssigkeit, misst aber gegen den üblichen, für alle anderen Röhrchen gültigen Blindwert mit 0.1 ml, liest die zugehörigen mg% auf der üblichen, für 0.1 ml gültigen Eichkurve ab und multipliziert anschliessend mit 1.04/2 (= 0.52).

Die Tabelle I beweist, dass auf diese Art innerhalb der methodischen Streuung volle Übereinstimmung der Resultate erzielt wird; die 2σ-Streuung der Differenz zwischen 43 auf beide Arten bestimmten identischen Proben beträgt nur $\pm$ 3.3 mg% (method. Streubereich = $\pm$ 4 mg%).

C. EICHKURVE

Wie in unserer früheren Publikation[1] erwähnt, verwenden wir zur Herstellung der Eichkurve nicht Normalliquores oder Serumverdünnungen, deren Eiweissgehalt mittels der Kjeldahlmethode bestimmt wurde, da auch dieser letzteren Methode gewisse Fehler anhaften. An deren Stelle gelangt bei uns ein Gemisch von 57% reinsten Albumin und 43% reinsten γ-Globulin, welche im Handel erhältlich sind, zur Verwendung. Es sei hier allerdings darauf hingewiesen, dass nicht alle, auch nicht die von der gleichen Herstellerfirma bezogenen Präparate stets denselben Reinheitsgrad aufweisen. Es empfiehlt sich darum, wenn z.B. Vergleiche in bezug auf die Höhe der Normaleiweisswerte im Liquor zwischen verschiedenen Laboratorien angestellt werden, auch jeweils die Extinktionsgrösse der zugrundeliegenden Eichkurven in Betracht zu ziehen.

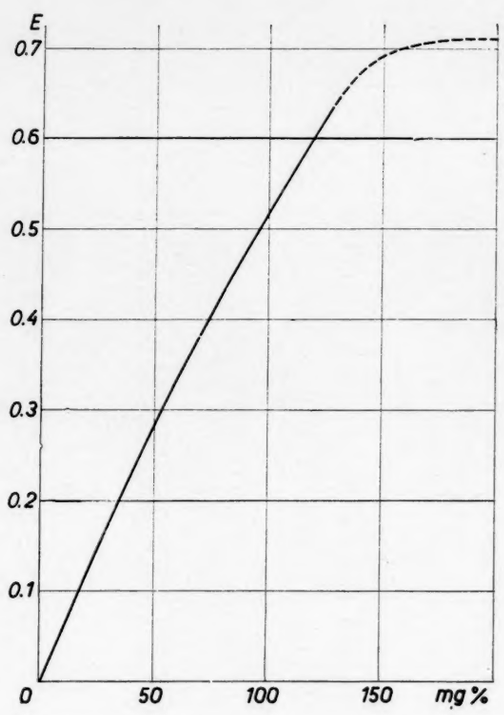

Fig. 1. Eichkurve zur Eiweissbestimmung mit der Folin–Cu-Methode (Alb./Glob. = 57/43).

Die Extinktion der von uns verwendeten Eichkurve ist durch die Werte der Fig. 1 festgelegt; sie ist durch 8-fache Bestimmung exakter Verdünnungsreihen gesichert. Die Angaben in mg% auf der Abszisse sind umgerechnet auf den *absoluten*

Literatur S. 740

Gehalt an *Reineiweiss*, nachdem sowohl beim Albumin wie beim Globulin-Präparat mittels verschiedener Methoden der Anteil von Wasser und anderen Begleitsubstanzen ermittelt worden war. So wurden oben genannte Präparate folgenden Analysen unterworfen:

(*a*) Gravimetrische Ermittlung des im Vakuum über P_2O_5 entziehbaren Wassers. Ferner wurden von der Lieferfirma die genauen Daten der üblichen Begleitstoffe der Albumin- und γ-Globulin-präparate angefordert und diese mit in Rechnung gestellt.

(*b*) Bestimmung der UV-Extinktion bei 280 mμ und Vergleich mit den Angaben der Behringwerke für ihre Reinst-Trockenpräparate.

(*c*) Bestimmung des Eiweissgehaltes nach dem Mikrokjeldahlverfahren von Abelin (Dr. LAUBER, Med.-chem. Inst., Bern).

(*d*) Bestimmung des Eiweissgehaltes nach dem Kjeldahlverfahren mit Kaliumsulfat und Cu-sulfat (Dr. SCHÖNIGER, Mikrolabor, Sandoz AG., Basel).

(*e*) Bestimmung des Eiweissgehaltes nach dem Verbrennungsverfahren über Cu von Dumas (Dr. SCHÖNIGER, Mikrolabor, Sandoz AG., Basel).

Die damit erzielten Ergebnisse stimmten im Rahmen der üblichen Streuung der einzelnen Methoden rel. gut überein. Wir gelangten auf diese Weise für unser Albumin/Globulin-Eichgemisch, bezogen auf 100% Reineiweiss, zu einer Extinktion im UV bei 280 mμ von $E^{1\%}_{1cm} = 9.47$ und zu einer solchen nach Bestimmung mittels dem Cu–Folin-Reagens bei 755 mμ von $E^{1\%}_{1cm} = ca.\ 300$ (d.h. infolge der leichten Krümmung der Kurve bei niedrigen Eiweisswerten etwas mehr, bei hohen etwas weniger; genauer ausgedrückt weist beispielsweise eine 100 mg%-ige Eiweiss-Eichlösung nach der Folin–Cu-Methode bestimmt eine Extinktion von $E = 0.517$ oder $E^{1\%}_{1cm} = 290$ auf, während eine gleiche 50 mg%-ige Lösung ein E von 0.284 oder $E^{1\%}_{1cm} = 318$ ergibt).

In vielen Dutzenden von Versuchen wurde festgestellt, dass das Extinktionsmaximum zwischen 760 und 750 mμ schwanken kann. Der kleinste Ablesefehler wird demnach bei 755 mμ gemacht. Um ein Versehen oder Verschreiben auszuschliessen, gehört es zu unserer Routine, jeweils auch die benachbarten Wellenlängen 740 und 770 mμ mitabzulesen. Die drei so erhaltenen Punkte müssen ja stets in einem mehr oder weniger gleichen Verhältnis zu einander stehen und Unstimmigkeiten sind sofort zu erkennen.

D. EINFLUSS DER TEMPERATUR AUF DIE FARBENTWICKLUNG

Im Zusammenhang mit der unter (A) geschilderten Abklärung von Fehlermöglichkeiten haben wir auch die Frage des Temperatureinflusses näher untersucht. Die mehrfache Bestimmung des gleichen Liquors unter verschiedenen Bedingungen führte zum Ergebnis der Tabelle II. Es ist sofort ersichtlich, dass bei 4° nach 1 1/2 bis 2 Stunden

TABELLE II

TEMPERATURABHÄNGIGKEIT DER FARBENTWICKLUNG

Farbentwicklung bei	E_{755} *gemessen nach Stunden*			
	1	1 1/2	2	2 1/2
4° (Eiskasten)	0.146	0.154	0.156	0.158
20° (Zimmertemp.)	0.159	0.159	0.159	0.159
30° (Brutschrank)	0.159	0.157	0.156	0.157

Literatur S. 740

der Endwert praktisch erreicht ist, bei Zimmertemperatur und darüber schon mindestens nach 1 Stunde.

Wir haben darum mit einem anderen Liquor weitere Versuche in rel. kühlen Räumen durchgeführt und zwar unter Verfolgung in kurzfristigen Intervallen. Das Resultat ist in Tabelle III zusammengestellt. Auch bei diesen niedrigen Temperaturen ist der Endwert innerhalb der Messgenauigkeit nach spätestens $1^1/_2$ Stunden erreicht.

TABELLE III

ZEITABHÄNGIGKEIT DER FARBENTWICKLUNG

E_{755} *nach Std.*	$^1/_4$	$^1/_2$	$^3/_4$	1	$1^1/_2$	2	$2^1/_2$
bei 15°	—	0.215	.0224	0.230	0.236	0.237	—
bei 17°	0.214	0.223	0.230	0.235	0.242	0.243	0.242

Es stellte sich daher die Frage, ob überhaupt – wie in der Originalmethode vorgeschrieben – ein zweistündiges Abwarten der Farbentwicklung notwendig ist, zumal man ja, wie Tabelle II zeigt, mit etwas Wärme nachhelfen kann.

Wir haben uns darum entschlossen, die folgende *Modifikation* einzuführen, bei welcher der Endwert schon nach 30 min erreicht ist: Nach dem Einpipettieren des Phenolreagens werden alle Röhrchen in ein *ca. 35° warmes Wasserbad* (Becherglas) gestellt und einfach sich selbst überlassen. Nach $^3/_4$ *Stunden*, während welcher Zeit

TABELLE IV

VERGLEICH DER NEUEN MODIFIKATION MIT DER ALTEN METHODE

C.S.F. Nr.	E_{755} *Methoden*		*Diff. in mE*	*C.S.F. Nr.**	E_{755} *Methoden*		*Diff. in mE*
	neue $^3/_4$ *Std.*	*alte 2 Std.*			*neue* $^3/_4$ *Std.*	*alte 2 Std.*	
72	0.111	0.112	+ 1	72	0.115	0.115	0
73	0.177	0.178	+ 1	74	0.143	0.148	+ 5
76	0.162	0.162	0	76	0.158	0.158	0
77	0.168	0.167	— 1	77	0.169	0.172	+ 3
78	0.160	0.159	— 1	78	0.162	0.159	— 3
79	0.217	0.216	— 1	79	0.222	0.221	— 1
80	0.166	0.169	+ 3	80	0.166	0.162	— 4
81	0.143	0.148	+ 5	81	0.145	0.144	— 1
82	0.177	0.181	+ 4	82	0.182	0.181	— 1
83	0.195	0.192	— 3	83	0.192	0.193	+ 1
79	0.238	0.238	0	82	0.188	0.188	0
85	0.627	0.626	— 1	85	0.605	0.615	+10
86	0.153	0.152	— 1	86	0.145	0.146	+ 1
84	0.254	0.254	0	85a**	0.228	0.230	+ 2
86	0.144	0.145	+ 1	87	0.160	0.159	— 1
88	0.100	0.110	+10				
Summe Diff.: (n = 16)			+17	(n = 15)			+11
			+11				
Total:			+28 : 31 = +0.9 mE durchschnittl. Diff. † (entspr. +0.2 ±0.62 mg%)				

* Vgl. Fussnote in Tab. I.

** Hier handelt es sich um eine 3-fache Verdünnung aus Nr. 85.

† Die durchschnittliche Differenz der beiden Methoden beträgt also nur rund 0.001 E (= 0.2 mg%), ihr Streubereich (± 2σ) nur ± 1.25 mg%; dieser ist somit kleiner als was nach der rein methodischen Streuung noch zulässig wäre. Der Unterschied ist folglich bedeutungslos.

Literatur S. 740

sich die Temperatur des Becherglases auf etwa 28° abgekühlt hat, wird in die Messküvetten übergegossen und 1–2 min später gegen den gleich behandelten Blindwert gemessen.

Eine Gegenüberstellung dieser neuen Modifikation und der bisherigen Methode wird in Tabelle IV gegeben. Es ist daraus ohne weiteres zu ersehen, dass bei Verwendung des vorgeschriebenen *Wärmebades* die *Versuchszeit unbedenklich und unter völliger Wahrung gleicher Exaktheit um etwa $^2/_3$ gekürzt, d.h. auf $^3/_4$ Std. festgesetzt werden kann.* Wir verwenden seither mit gutem Erfolg nur noch diese neue Ausführungsweise.

Theoretisch würde zwar unter obigen Bedingungen eine Wartezeit von einer halben Stunde schon genügen. Um jedoch im täglichen Routinebetrieb unabhängig zu sein vom Besitz geeigneter Thermostaten oder von der Notwendigkeit einer exakt einzuhaltenden und im Laufe der Wartezeit zu kontrollierenden Temperatur des Wasserbades (verschieden rasche Abkühlung je nach Zimmertemp.), geben wir als Sicherheitsmarge eine weitere Viertelstunde zu und gewinnen damit den Vorteil, dass eine *approximative* Temperatureinhaltung genügt. So haben wir uns in einer Serie von 11 Doppelversuchen überzeugt, dass es beispielsweise gleichgültig ist, ob die Wasserbadtemperatur zu Beginn der Wartezeit um 35° oder um 45° und am Ende der Wartezeit um 28° resp. noch über 30° betrug. Innerhalb der methodischen Streuung wurden auf beide Arten gleiche Resultate erhalten.

Es ist aber zu berücksichtigen, dass bei höheren Temperaturen die Gefahr des Flüssigkeitsverlustes durch Verdunsten grösser wird; ausserdem wäre nun als zusätzlicher Schritt im Arbeitsgang eine Abkühlung der Röhrchen auf Zimmertemp. kaum zu umgehen, damit nicht als neue Fehlerquelle Extinktionsdifferenzen infolge von ungleicher Temperatur der Messlösungen auftreten. Es empfiehlt sich also aus anderen Gründen, die obige Temperaturangabe von 35° nicht wesentlich zu überschreiten. Unter diesen Voraussetzungen wird nach Übergiessen in die Messküvetten und 1–2 min Stehenlassen (zwecks Entweichen allfälliger Luftbläschen) im allgemeinen eine Messtemperatur von rund 25° erreicht.

E. MODIFIZIERTE ARBEITSVORSCHRIFT

Auf Grund obiger Ergebnisse ist die Eiweissbestimmung in der zeitlich abgekürzten Form auf folgende Weise auszuführen:

I. Reagenzien

(A) 2% Na_2CO_3 in 0.1 *N* NaOH; vor Aufnahme von CO_2 schützen.
(B) 0.5% $CuSO_1$ $5H_2O$ in 1% Na-citrat.
(C) 1 ml Reagens B wird mit Reagens A auf 50 ml verdünnt. Die Mischung wird vor Gebrauch frisch angesetzt; sie ist nur beschränkt haltbar.
(D) Phenolreagens nach Folin–Ciocalteu, eingestellt auf 1 *N* Lösung (vgl. Details dazu in[2]).

II. Glaswaren und Behandlung

1. Nur hohe Reagensgläser verwenden.
2. Beim Schütteln darauf achten, dass ev. herausspritzendes Flüssigkeitströpfchen nicht an der Hand abprallen und wieder ins Röhrchen zurückfallen kann.

Literatur S. 740

3. Rand der Reagensgläser nicht mit den Fingern anfassen (Schweiss!), damit beim Übergiessen in die Messküvetten nicht Spuren von Eiweiss von dort mit hinübergespült werden.

III. Ausführung

Zu 5 ml Reagens C bringt man 0.1 ml Liquor und lässt 10 min stehen. Darauf wird unter sofortigem Schütteln 0.5 ml Phenolreagens D zugegeben. Nun stellt man die Probe in ein Wasserbad von 35° und überlässt sie während $^3/_4$ Std. sich selbst. Alsdann giesst man die benötigte Menge in die Küvetten über und misst gegen den Blindwert bei 755 mμ. Zum Blindwert wird anstatt der Eiweisslösung 0.1 ml physiol. NaCl gegeben.

IV. Vorsichtsmassnahmen und Auswertung

Siehe vorangehender Text, Kap. A–C.

DANK

Die Durchführung der vorliegenden Arbeit verdanken wir der Unterstützung durch den E. Barell-Fonds.

ZUSAMMENFASSUNG

Es wurde der Einfluss verschiedenster Bedingungen auf die Exaktheit der Eiweissbestimmung mittels der sog. "Folin–Cu-Methode" untersucht. Verschiedene Fehlerquellen und deren Vermeidung werden besprochen. Die Ergebnisse erlaubten eine zeitlich *abgekürzte Modifikation* einzuführen, deren Präzision in keiner Weise von der Originalmethode abweicht.

SUMMARY

IMPROVED METHOD OF DETERMINING PROTEINS WITH Cu(II) AND PHENOL REAGENT ACCORDING TO FOLIN–CIOCALTEU

The influence of various factors on the accuracy of protein determinations by the Folin–copper method was studied. Various sources of error and their avoidance were discussed. A modified method is suggested, which is more rapid without being less accurate.

LITERATUR

[1] H. P. RIEDER, *Clin. Chim. Acta*, 3 (1958) 455.
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NEURAMINIC ACID AND ITS RELATION TO CHRONIC BRONCHITIS II*

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In Part I it was shown that samples of sputum from chronic bronchitis patients contained up to five times the amount of sialic acid (now termed neuraminic acid) present in normal saliva. Evidence was presented to show that this neuraminic acid content was associated with the mucoprotein fraction. In continuing this work it was considered essential to attempt a more valid comparison *i.e.* to compare the saliva of chronic bronchitis patients with saliva from healthy subjects and to compare bronchial mucus (as a bronchial aspirate without any contaminating saliva such as occurs in the sputum) from bronchitic patients and healthy subjects. This attempt was confronted with difficulties because the bronchial mucus of a healthy subject is almost unobtainable since only minute amounts occur normally in the bronchus. Recourse was therefore made to bronchial mucus obtained during the operation of bronchoscopy from patients who did not suffer from chronic bronchitis.

MATERIAL AND METHODS

Source of materials

Bronchial mucus samples were collected by Mr. KENNETH MULLARD of Harefield Hospital, Middlesex, during the bronchoscopy of patients suffering from chronic bronchitis and other conditions. The apparatus used consisted of a polythene tube attached to a specially designed trap of about 1 ml capacity. The bronchial mucus samples, together with saliva samples from the same patients were forwarded to the Chemistry Department, Birmingham University and there treated as described below. The sputum samples were obtained from chronic bronchitics in a special unit for the treatment of chronic chest diseases set up at Benenden Hospital, Kent.

Initial treatment of materials

The sputum, mucus and saliva samples were each diluted with 2–3 volumes of distilled water and dialysed at 2° for 5 days with 2 changes of distilled water daily. After the dialyses were completed, the suspensions were freeze-dried and further dried to constant weight over P_2O_8 *in vacuo* at room temperature.

Bronchial mucus and saliva from chronic bronchitics

The neuraminic acid (NA) content of samples of bronchial mucus obtained during the bronchoscopy of patients suffering from chronic bronchitis was compared with that of their own saliva. The determination of neuraminic acid content was carried out according to the colorimetric method of SVENNERHOLM[1] which involves heating with orcinol reagent at 100° for 15 min. The results are given in Table I.

* Part I, *Clinica Chimica Acta*, 2 (1957) 491.

References p. 747

TABLE I

BRONCHIAL MUCUS AND SALIVA FROM CHRONIC BRONCHITICS

Subject	*Sex*	*Vol. (ml)*	*Non-dialysable material*		*mg NA/ml*
			Wt. from sample (mg)	*% Neuraminic acid*	
Bronchial mucus					
F.P.	M	1.3	22.73	2.45	0.427
C.T.	M	0.4	25.99	2.09	1.36
T.M.J.	M	0.5	19.40	2.13	0.83
S.P.	M	0.6	20.10	3.21	1.08
Saliva					
F.P.	M	1.5	8.15	0.02	0.001
C.T.	M	1.0	7.36	0.25	0.018
T.M.J.	M	7.8	25.62	0.05	0.002
S.P.	M	7.7	68.30	0.00	0.00

Brief case histories are as follows:

F.P. (aged 56). 1916—pneumonia. He had suffered troublesome symptoms of bronchitis since 1952. On examination there was evidence of moderately severe bronchitis and emphysema. Bronchoscopy showed no abnormality other than that of bronchitis.

C.T. (aged 38). 1936—inhaled a tin-tack (upholsterer). 1938—haemoptysis—tin-tack removed. Bronchogram—no bronchiectasis. 1942—pneumonia. Had progressive cough, dyspnoea and wheeze since then and examination revealed bronchitis and emphysema. Bronchoscopy showed no abnormality other than that of bronchitis.

T.M.J. (aged 55). Admitted to no chest symptoms prior to 1957. Since then he had suffered recurrent bronchitis. On examination chronic bronchitis and emphysema diagnosed suggesting a longer history. Bronchoscopy showed no abnormality other than that of bronchitis.

S.P. Had a 10 year history of bronchitis, persistent throughout the year for the last 3 years. Bronchoscopy showed no abnormality other than bronchitis and emphysema.

Bronchial mucus and saliva from controls

The neuraminic acid content was determined as above on bronchial mucus obtained during bronchoscopy of "controls" and compared with that of their own saliva. The results are given in Table II.

TABLE II

BRONCHIAL MUCUS AND SALIVA FROM CONTROLS

Person	*Sex*	*Vol. (ml)*	*Non-dialysable material*		*mg NA/ml*
			Wt. from sample (mg)	*% Neuraminic acid*	
Bronchial mucus					
E.T.	F	0.5	9.98	1.91	0.38
F.D.	M	0.2	3.50	0.93	0.17
F.D.*	M	0.4	24.54	1.27	0.78
P.C.	M	0.6	10.66	0.63	0.11
M.L.	F	0.4	15.46	0.99	0.38
E.R.	F	0.4	23.14	1.80	1.04
F.S.	F	0.3	14.34	1.67	0.80
Saliva					
E.T.	F	0.9	28.02	0.03	0.009
F.D.	M	1.4	4.79	0.05	0.002
P.C.	M	0.6	3.92	0.49	0.032
M.L.	F	0.5	4.30	0.20	0.017
E.R.	F	3.4	2.40	0.21	0.002
F.S.	F	3.2	5.43	0.04	0.001

* Note: This sample was contaminated with blood.

References p. 747

Brief case histories are as follows:

E.T. (aged 54). Bronchoscopy carried out because she swallowed a damson stone which she felt was lodged in her throat. No abnormality found on bronchoscopy. Chest X-ray showed a lesion in the left zone, old tuberculosis?

F.D. (aged 54). Pneumonia with effusion. One month previously had blood-stained sputum. Pleural thickening found only at right base. Bronchoscopy N.A.D.

P.C. (aged 39). Hiatus hernia. Bronchoscopy carried out following oesophagoscopy. Bronchial tree normal.

M.L. (aged 56). Probably had new growth of the oesophagus. Bronchoscopy carried out after oesophagoscopy. Bronchial tree normal.

E.R. (aged 51). Hiatus hernia. Bronchoscopy carried out after oesophagoscopy. Bronchial tree normal.

F.S. (aged 57). Hiatus hernia. Bronchoscopy carried out after oesophagoscopy. Bronchial tree normal.

Bronchial mucus from patients with other conditions

Samples of bronchial mucus were obtained during the bronchoscopy of two patients (A and B, see Discussion) suffering from other conditions. The results of neuraminic acid determination are given in Table III.

TABLE III

BRONCHIAL MUCUS FROM PATIENTS WITH OTHER CONDITIONS

Mucus	*Patient*	*Sex*	*Volume (ml)*	*Non-dialysable material*		*mg NA/ml*
				Wt. from sample (mg)	*% Neuraminic acid*	
A	J.D.	M	0.50	29.0	1.43	0.83
B	C.T.	M	0.60	27.5	2.04	0.94

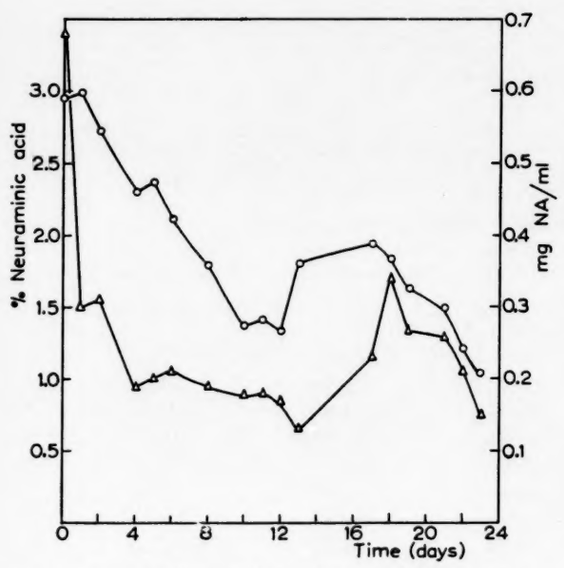

Fig. 1. Variation with time of the amount of neuraminic acid in the sputum of an improving chronic bronchitis patient. ○—○ % NA. △—△ mg NA/ml.

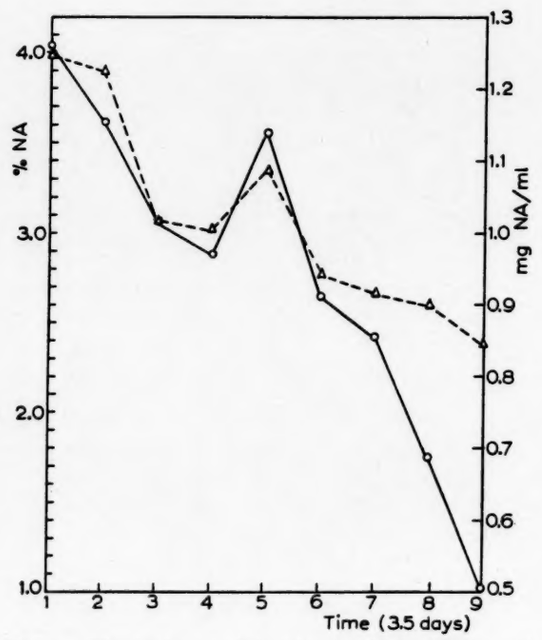

Fig. 2. Variation with time of the amount of neuraminic acid in the sputum of an improving chronic bronchitis patient △—△ % NA; ○—○ mg NA/ml.

References p. 747

Neuraminic acid content of sputum from improving chronic bronchitis patients

(1) *Daily samples.* Samples of sputum were collected at 8 a.m. daily from a male patient (N.T.) age 53 yrs. who had suffered from chronic bronchitis for 16 yrs. Samples were treated as above and their neuraminic acid contents determined—all samples produced a chromogen having peak absorption at 575 ± 5 mμ. The results are given in Table IV and plotted in Fig. 1. The results of hexosamine determinations[2, 3] are also included.

(2) *3-day samples.* Samples of sputum produced during three days by a male chronic bronchitic (E.C., age 57 yrs.) were pooled together and this was repeated over a period of weeks. Each lot of bulked sputum was treated in the usual way. The results of the neuraminic acid determinations are given in Table V and shown graphically in Fig. 2.

TABLE IV

SPUTUM SAMPLES (24 h) FROM A CHRONIC BRONCHITIC

Date	*Volume (ml)*	*Non-dialysable material*		*mg NA/ml*	*% Hexosamine*
		Wt. from sample (mg)	*% NA*		
7.2.58	2.7	62.2	2.94	0.68	10.40
8.2.58	6.5	65.6	2.99	0.33	—
9.2.58	5.4	63.7	2.72	0.32	—
11.2.58	14.5	122.0	2.29	0.19	—
12.2.58	16.5	140.8	2.37	0.20	13.69
14.2.58	19.5	194.0	2.11	0.21	—
16.2.58	17.5	184.8	1.80	0.19	5.30
19.2.58	10.5	135.2	1.37	0.18	—
20.2.58	9.0	119.3	1.39	0.18	6.74
22.2.58	7.0	90.2	1.34	0.17	—
23.2.58	8.7	62.2	1.80	0.13	12.17
27.2.58	10.0	117.1	1.94	0.23	—
28.2.58	11.5	210.2	1.84	0.34	7.99
1.3.58	7.1	114.4	1.62	0.26	—
3.3.58	11.2	201.5	1.49	0.27	—
4.3.58	7.0	123.0	1.22	0.21	4.37
5.3.58	5.0	81.8	1.04	0.17	—

TABLE V

SPUTUM SAMPLES (3 DAYS) FROM A CHRONIC BRONCHITIC

Date	*Volume (ml)*	*Non-dialysable material*		*mg NA/ml*
		Wt. from sample (g)	*% NA*	
21.4.58–24.4.58	154	4.8622	3.99	1.260
25.4.58–28.4.58	135	3.9683	3.90	1.152
29.4.58– 1.5.58	75	2.4885	3.07	1.018
2.5.58– 5.5.58	67	2.1549	3.02	0.971
6.5.58– 8.5.58	42	1.4368	3.35	1.146
9.5.58–12.5.58	61	2.0072	2.77	0.912
13.5.58–15.5.58	19	0.6104	2.67	0.858
16.5.58–19.5.58	24	0.6359	2.60	0.689
20.5.58–22.5.58	42	0.8880	2.39	0.505

References p. 747

Hexosamine contents of saliva, mucus and sputum

A weight of material estimated to contain about 1 mg of hexosamines was hydrolysed for 18 h with 2 *N* HCl (2 ml) in a sealed tube at 100°. The total hexosamine content of an aliquot of the hydrolysate was determined by the method of SVENNERHOLM[2] after prior removal of amino acids and interfering chromogens by passage down a column of Dowex-50 (200–400 mesh) (H^+) as described by BOAS[3]. Determinations were carried out on normal saliva from four healthy subjects P.M.G., R.B.W., B.P., and M.G., bronchial mucus obtained during bronchoscopy of cases A and B—see above and finally from two chronic bronchitis patients (H.C. and E.K.). The results are given in Table VI.

Quantitative determinations of individual hexosamines present in some of the samples was effected on ion exchange colums calibrated against authentic D-glucosamine and D-galactosamine as described by GARDELL[4]. The results obtained are also given in Table VI.

TABLE VI

HEXOSAMINE CONTENTS OF SALIVA, MUCUS AND SPUTUM

Subject	*Sex*	*Total hexosamine* %	*Glucosamine* %	*Galactosamine* %
P.M.G.	M	3.39	2.79	0.60
R.B.W.	M	5.81	—	—
B.P.	F	5.36	—	—
M.G.	F	3.62	—	—
J.D. (Case A)	M	6.03	—	—
C.T. (Case B)	M	6.70	4.80	1.80
H.C.	M	10.54	8.01	2.54
E.K.	M	9.86	7.71	2.14

Hexose contents of saliva, mucus and sputum

A weight of material containing 50–400 μg of hexoses was weighed directly into a stoppered tube and 3 *N* H_2SO_4 (2 ml) added. After hydrolysis at 100° for 2 h the hexose content was determined by the method of SVENNERHOLM[5]. When determined in this way saliva from P.M.G. contained 9.96% hexoses, mucus from C.T. contained 10.13% and bronchitis sputum from E.K. contained 10.91% hexoses calculated as galactose.

DISCUSSION

With bronchitics and controls the neuraminic acid content of the bronchial mucus was on average 30 and 8 times greater respectively on a dry weight basis (see Tables I and II) than that of their salivas. Although there was little or no difference in the neuraminic acid content of saliva from chronic bronchitics and that from healthy subjects, there was a definite trend in the few cases investigated to higher values in the bronchial mucus of bronchitics than in that of controls.

It should be mentioned that the neuraminic acid content of saliva is difficult to determine accurately and the figures given in Tables I and II represent maximum values. This is because the neuraminic acid content in saliva is so small that inter-

References p. 747

ference from chromogens produced by other sugars, which was negligible in the case of sputum, becomes more prominent with saliva. Only a few specimens of saliva produced a chromogen with a maximum at 575 mμ and even in these the absorption was just an arrest in the curve.

It was of interest to determine the neuraminic acid of bronchial mucus from patients suffering from other conditions. Case (A) was a man who had had a pneumonitis which had cleared radiologically by the time of admission to hospital. His bronchial mucus neuraminic acid content (1.43%) fell into that of the "controls" (*cf.* Tables II and III). Case (B) was a man with a bronchial carcinoma who had also been in congestive cardiac failure and had deep vein thrombosis of the leg. His bronchial mucus neuraminic acid content (2.04%) fell into that of the bronchitics (*cf.* Tables I and III). Such results indicated that the neuraminic acid content of bronchial mucus could not of itself be used in the diagnosis of chronic bronchitis.

It was therefore decided to attempt to follow the recovery of a bronchitis patient through the neuraminic acid content of his sputum since the actual proportion of bronchial mucus present in such sputa would decrease as he progressed. Such a recovery pattern was determined on two such patients. In the first of these experiments sputum samples, collected at 24 h intervals, showed a remarkable decrease in their neuraminic acid contents as the condition of the patient improved (see Table IV). An overall decrease from 2.94% to 1.04% (% by weight) was observed over an interval of only 26 days. The actual content of neuraminic acid (NA) per ml of sputum exhibited a variation of roughly the same tendency. The decrease however, in both percentage by weight and mg NA/ml was not smooth (Fig. 1). A second patient whose total output of sputum over 3–4 day intervals was collected showed a relatively smoother but less sharp decrease as his condition improved (see Table V). The neuraminic acid content (% by weight) decreased from 3.99% to 2.39% over an interval of 31 days. The actual content in mg/ml showed a steeper decrease (Fig. 2; from 1.260 to 0.505) and was more evident than the corresponding one shown by the first patient (from 0.68 to 0.17). The total output of neuraminic acid over three days also became less as the condition of the patient improved. Such findings give a chemical method of tracing the recovery of a chronic bronchitis patient.

An attempt was then made to find whether the hexosamine content would prove of similar value. The method adopted for the determination of hexosamine was the SVENNERHOLM[2] modification of the Elson-Morgan method. Since amino acids and sugars interfere in this reaction the hydrolysates of the samples were chromatographed on cation-exchange resins according to the method of BOAS[3]. Hexosamine determinations on the sputa of the first of the above two improving chronic bronchitis patients showed no regular pattern. One explanation of this may be that two types of mucoprotein are present, one containing only hexosamine and the other both hexosamine and neuraminic acid. Under these conditions the production or disappearance of the latter could only be followed by neuraminic acid determination especially if the hexosamine contents of both types of mucoproteins were similar. However the possibility existed that the nature of the hexosamine (whether glucosamine, galactosamine, etc.) varied in two such mucoproteins. Hence the nature and amount of each hexosamine present in sputum, mucus and saliva was determined by separation on a calibrated ion-exchange resin[4]. In every case investigated only glucosamine and galactosamine were detected in the hydrolysates. The first was invariably

References p. 747

the predominant constituent but the ratio glucosamine/galactosamine varied: 2.7:1 in mucus (total hexosamine content, 6.70% by weight), 3.6:1 in sputum (total 9.86%) and 4.7:1 in saliva (total, 3.39%). The fact that the ratio is smaller in mucus than saliva may be of some physiological significance. The corresponding hexose contents (calculated as galactose) were mucus, 10.13%; chronic bronchitis sputum, 10.91% and normal saliva, 9.96%. The above figures for hexosamines and hexoses in saliva might include in them those present in the blood-group substances[6] since about 80% of humans secrete blood-group substances in their saliva.

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SUMMARY

Comparisons have been made of the neuraminic acid, hexosamine and hexose content of saliva and bronchial mucus obtained from chronic bronchitis patients and controls. It has been shown that a bronchitic gives a bronchial mucus, which contains on average 30 times more neuraminic acid (per g of non-dialysable material) than is present in his saliva. With controls the neuraminic acid content of their bronchial mucus was on average 8 times greater than that of their saliva. Since the proportion of bronchial mucus in a chronic bronchitic's sputum decreases as he recovers, a patient's progress can be followed chemically by neuraminic acid determination.

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THE USE OF RADIO-SULPHUR IN THE ANALYSIS OF PROTEIN RESORPTION IN THE INTESTINES*

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Knowledge of the physiology of protein resorption is based mainly on experiments in metabolic balance and on investigations in men and animals with fistulas. It could be examined more closely by the use of radio-isotopes in animal experiments. In principle, a protein marked by radio-isotope is used as a test food, and the radioactivity is measured at different times in the contents of the bowels, in the intestinal wall and in the venous intestinal blood. The chemical nature of each measured radioactive compound can be defined by means of microbiological, chromatographic, electrophoretic and by precipitation methods. Autoradiographic examination of histological slides reveals a relationship between the activity found and the morphology of the cells of the intestinal wall. By the use of radio-isotopes, the location of the protein resorption and the role of the intestinal wall in protein metabolism can be studied.

In the present investigations we used radio sulphur (^{35}S), whose radiant energy and half-life make it suitable for biological investigations. As test food we used protein from yeast labelled with ^{35}S; this was prepared by growing *Torula utilis* in a nutritive solution containing traces of highly active ^{35}S instead of usual sulphur (SCHLÜSSEL[1]). Thus, nearly 100% of the available activity is used and a highly active, biologically marked protein is obtained, and the application of the sufficient test food strains the intestinal tract no more than in its state of physiological rest. Different activities can be obtained by addition of the same inactive material. It is also possible to get a ^{35}S labelled hydrolysate by hydrolysing the yeast protein; hence the test food can be applied not only orally, but also rectally and parenterally.

METHODS AND RESULT

Location of the protein resorption

To discover where the resorption takes place, 1 ml of ^{35}S-test food was applied orally and rectally by a stiff probe to young female white rats. At intervals of 15 min, 30 min, 1, 2, 4, 6, 8 and 10 h after the application, 5 animals, living under the same conditions, were killed. The gastrointestinal tract was divided into 10 parts and the activity was measured.

In Fig. 1 are shown the average values of the activity in the intestinal wall related to the same dry weight of organs as found 15 min after oral and rectal application. Those parts of the intestines in direct contact with the ^{35}S-test food have a higher activity. Similar results can be obtained in each part of the intestinal tract. Therefore it can be assumed that the wall of the whole gastrointestinal tract is able to resorb.

Under physiological conditions, the resorption after oral protein intake takes place in the duodenum and the small intestine. This assumption is justified by a com-

* Aided by grants from the Deutsche Forschungsgemeinschaft.

References p. 751

parison of the curves obtained after oral, rectal and parenteral application of ^{35}S-test food (Fig. 2). The points shown are the amounts of activity related to the same organ dry weight. For each part of the intestinal wall all the activities measured at the single times of killing mentioned above were added. From Fig. 1, the accumulation of

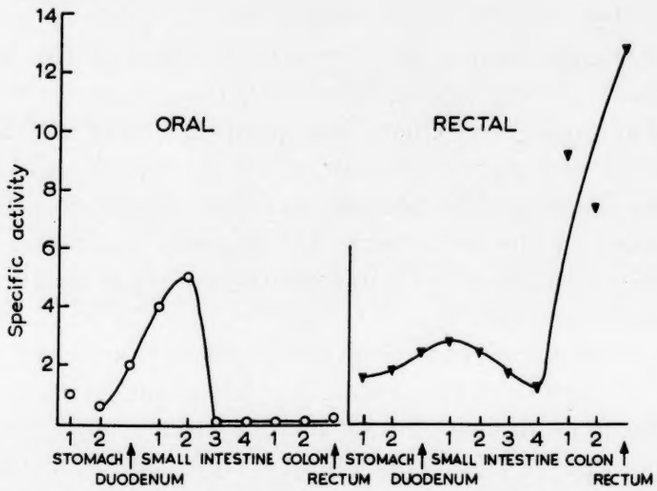

Fig. 1. Rat intestines. Application of ^{35}S-labelled yeast-protein and yeast-hydrolysate.

activity is highest in those parts of the intestinal wall which had direct contact with the test food. Only the line of the intraperitoneal application is not influenced by this accumulation of activity due to resorption. In this case, the same high specific activity of protein products was shown for a short time throughout the gastrointestinal tract

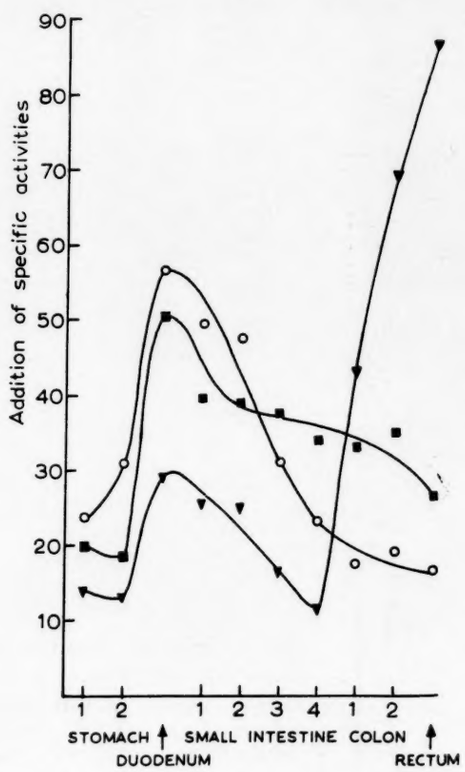

Fig. 2. Rat intestines. Application of ^{35}S-labelled yeast-protein or yeast-hydrolysate. O——O oral; ▼—▼ rectal; ■—■ i.p.

References p. 751

by the blood. From the difference between the data in oral and intraperitoneal application, it can be seen that the points of contact between the test food and the intestinal wall occur in the upper part of the gastrointestinal tract, which means that the protein resorption takes place essentially in those parts.

The role of the intestinal wall in protein metabolism

It is remarkable that such a high activity is found in the intestinal wall. The movement of marked protein products from the intestinal lumen into the bloodstream is only transitional and does not explain the accumulation of activity. However, there must be an important protein metabolism of the intestinal wall. Some experimental results indicate the formation of proteic secretion in the intestines. TARVER AND SCHMIDT[2] found most of the activity in the mucous membrane of the intestines. By autoradiography, NIKLAS AND OEHLERT[3] showed that nearly all the activity is found in Lieberkuhn's crypts.

The frequent assumption that the intestinal wall takes part in the formation of the serum protein has not been confirmed. The latest report on the subject is that of MILLER *et al.*[4], who made investigations in rats after application of lysine labelled with ^{14}C. We tried to prove the formation of serum protein in the intestinal wall of dogs and rabbits. From an isolated loop of the small intestine venous blood was taken (without loss) during resorption of ^{35}S-labelled yeast protein, and the serum protein was analysed by electrophoresis. By autoradiography of the electrophoresis paper, it was found that much of the absorbed protein products migrated together with the fractions of serum protein.

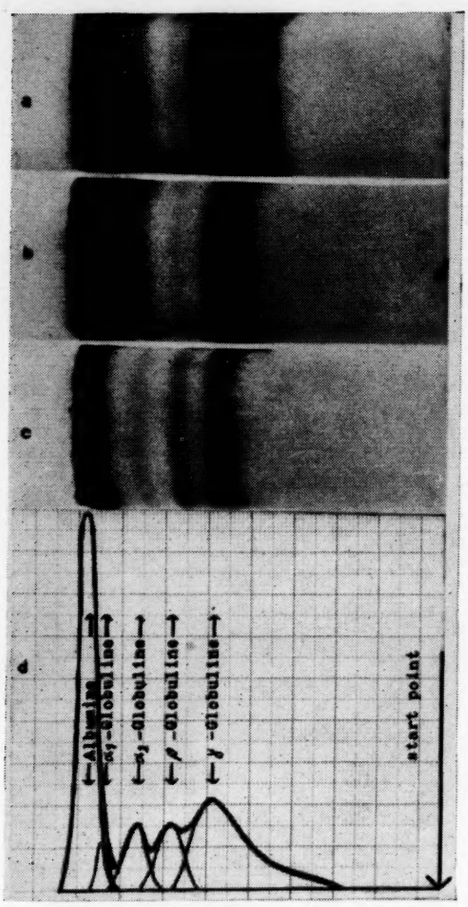

Fig. 3. Venous blood from the small intestines (rabbit), 8–10 min after the start of resorption of ^{35}S-labelled yeast-protein (serum differentiated by paper electrophoresis). a. Autoradiogram of an unstained electrophoresis slip. b. Autoradiogram of the slip after staining and washing out. c. Electrophoresis paper, protein fractions dyed with amido black. d. Relative contents of protein in the protein fraction dyed with amido black.

References p. 751

The fractional precipitation of protein also showed a radio-marking of albumen and globulines, but we found the same results after adding a hydrolysate of ^{35}S-yeast protein to blood serum *in vitro*. Thus it can be assumed that the marking of serum protein fractions found is not a real formation of serum protein, but that part of the resorbed ^{35}S-amino acids is absorbed on the serum proteins. Moreover, the results show that the important protein metabolism of the intestinal wall is only explained by the formation of the intestinal secretion. The amino acids necessary for this are taken from the blood and intestinal contents by the mucous membrane of the intestines.

SUMMARY

The resorption of yeast-protein in the rat takes place in the duodenum and the small intestine, after rectal application in the colon. The formation of intestinal secretion explains the important role of the intestinal wall in protein metabolism, the amino acids used are taken from the blood and directly from the intestinal contents. In an isolated loop of the small intestine (dog, rabbit) the formation of serum protein could not be proved. The resorbed amino acids are absorbed on the serum proteins differentiated by paper electrophoresis.

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SHORT COMMUNICATION

Plasma amino acids in experimental lathyrism

The urinary excretion of tyrosine, taurine and histidine was relatively increased in the experimental lathyrism[1]. A green spot was observed in the chromatograms and was identified as β-aminopropionitrile, which is also confirmed by STRONG *et al.*[2] This note on the plasma amino acids is meant to supplement the information on their urinary excretion.

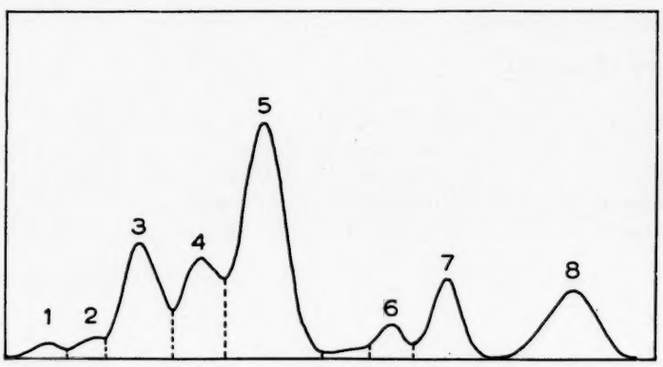

Fig. 1. The pattern of the densitometric evaluation of one-dimensional chromatograms of the amino acids from rat plasma. The statistically significant changes in experimental lathyrism are listed in Table I.

References p. 752

The feeding of rats was essentially the same as described before. The signs of osteolathyrism were confirmed by X-ray examination. Since it was found that the addition of iproniazid aggravates the symptoms[3], the animals received about 0.8 mg of Marsilid "Roche" in the daily diet, which contained 75% sweet pea meal.

The blood was collected by heart puncture under ether anesthesia. After centrifugation the proteins were precipitated with an excess of 80% ethanol. The supernate was obtained by filtration and evaporated on a boiling water bath. The residue was dissolved in acetone (containing 1% concentrated hydrochloric acid, v/v) and applied as spots containing about 0.1 ml plasma. One-dimensional chromatograms were run with butanol–acetic acid–water (4:1:5) as solvent and stained with ninhydrin. The chromatograms were evaluated quantitatively with a densitometer (Joyce, Loebl & Co., Ltd., Model 3 SR). The peaks (Fig. 1) were cut off and their areas determined

TABLE I

COMPARISON OF SOME AMINO ACID PEAKS

Peak number and identification (Fig. 1)	*Control rats (7) mean* %	*Lathyritic rats (6) mean* %	*t*	*P*
1. Cysteic acid	1.2[1]	0.6[1]	7.07	< 0.001
2. Basic amino acids	2.7	1.9	—	—
6. Tyrosine	3.6	3.0	3.17	< 0.01

[1] of total ninhydrin colour.

by weighing. The composition of the spots was ascertained by means of two-dimensional chromatograms with phenol as the second solvent. Statistically significant differences are listed in Table I. No green spot was detected and spot "No. 6" which was present in the urine of lathyritic rats was not obtained.

Considering the earlier information, it seems that the loss of tyrosine, sulphur-containing amino acids and histidine is due to a renal cause. β-Aminopropionitrile is not retained by the kidneys.

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CONTENTS

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